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HEAT STABLE EXTRACELLULAR ENZYMES OF *PSEUDOMONAS*

by

Ramarathna Koka

A dissertation submitted in partial fulfillment  
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY  
Logan, Utah

1999



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## ABSTRACT

Heat-Stable Extracellular Enzymes of *Pseudomonas*

by

Ramarathna Koka, Doctor of Philosophy

Utah State University, 1999

Major Professor: Dr. Bart C. Weimer  
Department: Nutrition and Food Sciences

Psychrotrophic bacteria produce heat-stable lipase, protease, and phospholipase. Previous studies indicate the production of multiple enzymes in several strains of *Pseudomonas fluorescens*, but conclusive evidence is lacking.

The influence of culture conditions on the production and thermostability of phospholipase, protease, and lipase was investigated in 17 raw milk and environmental isolates. Production and thermostability of the enzymes were influenced by strain, stage of growth, and the culture medium. Cross-reactivity of antibodies raised to a purified protease and a commercial lipase indicated the immunological diversity of the enzymes.

Protease purification was undertaken to investigate the production of multiple proteases within a single strain. A single monomeric protease with a molecular weight of 52 kDa was purified from *P. fluorescens* RO98. Biochemical characterization of the enzyme revealed that it was a zinc-metallo acidprotease with pH and temperature optima of 5.0 and 35°C, respectively. The enzyme was thermostable with a  $D_{55}$  of 41 min and a  $D_{62.5}$  of 18 h.

Although sensitive assays exist for proteases, they are not suitable for detection of protease activity in milk in the presence of milk proteins. Existing immunoassays approach the required sensitivity but take about 6 h and cannot distinguish between active and inactive enzyme. An immunoassay that can be completed within 2 h and that can detect and distinguish both total and active enzyme was explored. The ratio of these two forms gives insight into the history of the milk.

The ability of the purified protease to hydrolyze hydrophobic peptides associated with bitterness in Cheddar cheese was also investigated. Results demonstrated that the protease had the potential to debitter Cheddar cheese because it was able to hydrolyze the bitter peptides commonly found during aging.

Two lipolytic enzymes with molecular weights of 50 (Pf-lip1) and 12 kDa (Pf-lip2) were purified from *P. fluorescens* RO98. Differences were observed in their biochemical properties.  $D_{62.5}$ -values of 12.7 and 29.9 h were determined for Pf-lip1 and Pf-lip2, respectively. Pf-lip1 preferred longer chain length fatty acids, and Pf-lip2 preferred shorter chain length substrates. Pf-lip1 hydrolyzed milk fat and emulsified triolein, but Pf-lip2 did not, indicating that the latter was an esterase. This information is of significance to the dairy industry because activity tests that assay both the lipolytic enzymes need to be used in order to direct raw milk to short shelf-life products during processing and ensure quality of long shelf-life products.

(228 pages)

## ACKNOWLEDGMENTS

The most onerous task in completing this task is acknowledging support received from various sources. I realize that I may have excluded someone, this by no means discounts their contribution.

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Ramarathna Koka

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## LIST OF SYMBOLS, NOTATIONS, DEFINITIONS

ADH = Adenine dihydrazine

BCA = Bicinchoninic acid

BSA = Bovine serum albumin

BSTFA = N,N-bis(trimethylsilyl)trifluoroacetamide

CFU = Colony forming units

D<sub>55</sub> = D-value at 55°C

D<sub>62.5</sub> = D-value at 62.5°C

EDTA = Ethylene diamine tetraacetic acid

ELISA = Enzyme linked immunosorbent assay

FFA = Free fatty acids

GC = Gas chromatography

HRP = Horseradish peroxidase

HTST = High temperature short time

IMB = Immunomagnetic beads

LSD = Least significant difference

LTi = Low temperature inactivation

LTLT = Low temperature long time

MFGM = Milk fat globule membrane

OPA = *o*-Phthialdehyde

PBST = PBS containing 0.1% Tween 20

Pf-lip1 = *Pseudomonas fluorescens* lipase 1

Pf-lip2 = *Pseudomonas fluorescens* lipase 2

PLC = Phospholipase C

PLC-H = Phospholipase C -Hemolytic

PLC-N = Phospholipase C -Nonhemolytic High

RFU = Relative fluorescence units

TCA = Trichloroacetic acid

TMB = 3, 3', 5, 5' - Tetramethyl benzidine

TMCS = Trimethylchlorosilane

UHT = Ultra-high temperature

## CHAPTER I

### INTRODUCTION

Widespread use of refrigeration on farms and in milk processing plants improves milk quality by inhibiting the growth of many mesophilic and coliform bacteria, but selects for the growth of psychrotrophic bacteria (46, 81). Psychrotrophic bacteria possess a great potential for production of metabolites that contribute to spoilage before and after processing of milk (29). Although conventional heat treatment of milk by pasteurization and ultra-high temperature (UHT) pasteurization destroy psychrotrophic organisms, these organisms are important because they produce heat-stable metabolites prior to the heat treatment and are common post-processing contaminants. These metabolites are primarily extracellular lipase, protease, and phospholipase that withstand conventional heat treatment, remain active during storage, and degrade milk constituents leading to flavor and functionality defects (32). High numbers of psychrotrophic bacteria in raw milk cause off-flavor defects in fluid milk and dairy products such as butter, cream cheese, and UHT products as a result of liberation of free fatty acids by lipolysis and physical defects such as gelation due to proteolysis during storage. Therefore, the quality of fluid milk as well as products made from it is adversely affected (29).

Lipases and proteases exhibit different biochemical properties depending on growth conditions. With lipase, these observations may be explained by a number of underlying parameters. The enzyme may assume multiple tertiary or quarternary conformations or isozymes with differing heat resistance; changes may be produced simultaneously during logarithmic

growth, or a new set of lipases may be expressed during the stationary phase (41, 55, 166). The exact number of lipases produced by pseudomonads is not known, nor are the environmental parameters that govern their expression. Previous studies indicate the production of multiple lipases in several strains of *Pseudomonas fluorescens* but conclusive evidence is lacking. The optimum activity characteristics of lipases from the same species are diverse in their characteristics. The optimum pH range for lipase preparations is 7 to 9; optimum temperature is 30 to 70°C; molecular weight ranges from 33 to 50 kDa; and the enzyme exhibits highly variable heat inactivation values (43, 55, 80). The variability in these parameters indicates that multiple enzymes may be produced by a single strain.

Previous attempts to purify these enzymes suggest that multiple enzymes are produced, as indicated by the production of multiple activity peaks during chromatographic separation of extracellular proteins of a *Pseudomonas* culture. Most of the workers, however, purified and characterized the major activity peak ignoring the other peaks (42, 55).

Production of multiple proteases is also suspected (102, 157). Most of the biochemical properties of these enzymes were similar, including molecular weights. Limited information is available concerning these enzymes, including lack of amino acid sequence information; hence, isolation of the same enzyme cannot be ruled out in these studies.

Although it is suggested that more than one lipase and protease is produced by a species and is dependent on growth media and environmental growth conditions, it has not been confirmed by traditional enzymological techniques. Production of multiple enzymes with different properties further intensifies the problem of these enzymes in milk because their detection and

control become difficult.

The objective of this study was to determine if multiple lipases and proteases were produced in *P. fluorescens* RO98. This involved a study of the influence of growth conditions on the production and thermostability of lipase, phospholipase, and protease. Diversity of protease and lipase produced by some raw milk isolates was explored using immunological cross-reactivities. Production of multiple enzymes in a selected strain was investigated by purification and characterization of protease and lipase.

The influence of culture conditions for phospholipase production in various raw milk and environmental isolates of *Pseudomonas* is discussed. Each strain was studied for production of phospholipase during a 50 h incubation period in four media. Thermostability of the enzyme under low temperature long time (LTLT) pasteurization conditions (62.5°C for 30 min) was also studied.

The influence of culture conditions on protease production is discussed. The objective of this portion of the study was to determine if multiple proteolytic enzymes with differences in thermostability were produced in the strains studied. Isolation and characterization of the protease produced in *P. fluorescens* RO98 investigated production of more than one protease within a single strain.

An immunoassay for the determination of protease activity was explored, and the details of this work are described. The ability of a protease isolated from *P. fluorescens* RO98 to hydrolyze peptides associated with bitterness in Cheddar cheese is presented.

The production of multiple lipases among the isolates of *Pseudomonas* was investigated. The influence of culture conditions on production and

thermostability of lipases by the isolates was noted. Immunological diversity of the lipases among the isolates is also described. Isolation and characterization of lipolytic activity are detailed.

## CHAPTER II

### LITERATURE REVIEW

#### PSYCHROTROPHIC BACTERIA CONTAMINATING RAW MILK

About 15 genera of psychrotrophic bacteria have been isolated from milk and dairy products. These are mainly Gram-negative bacteria belonging to the genera *Pseudomonas*, *Achromobacter*, *Alcaligenes*, and *Enterobacter* (90, 123, 147, 170). Thermotolerant, psychrotrophic bacteria, such as *Bacillus*, *Streptococcus*, *Lactobacillus*, and *Enterococci*, survive pasteurization and grow in milk at refrigeration temperatures during storage and can cause spoilage (164). However, *Pseudomonas* predominates in milk stored at low temperatures before pasteurization. Additionally, these bacteria are common post-processing contaminants. The predominant lipolytic species in raw milk are the fluorescent pseudomonads (149). Within fluorescent pseudomonads, *Pseudomonas fragi* and *Pseudomonas fluorescens* dominate in milk stored at low temperatures and can cause defects (32, 149).

The International Dairy Federation defines a psychrotroph as a microorganism that grows at or below 7°C, irrespective of the optimum growth temperature (164). Raw milk is commonly held at refrigeration temperatures for periods of 2 to 3 d before processing, which allows many types of psychrotrophic bacteria to quickly grow and reach  $10^5$  to  $10^7$  colony forming units/ml (cfu/ml). A psychrotrophic bacterial load between  $10^2$  to  $10^8$  cfu/ml is detrimental to raw milk quality (29). Large microbial populations are achieved in 3 to 5 d with a generation time of 8 h at 4°C producing a significant amount of degradative enzymes (32, 153). Although the bacteria do not survive heat processing, most of the enzymes produced are heat-stable

and remain active in the finished product, which affects the acceptability of milk and milk products (Table 1). As a consequence of growth of psychrotrophic bacteria and secretion of extracellular, thermostable enzymes, a reduced shelf-life of dairy products is produced (27). A shelf-life of only 10 to 14 d is achieved in UHT milk processed from raw milk heavily contaminated with *P. fluorescens* compared with 56 d for that of high quality milk (97).

Defects associated with thermostable enzymes can be minimized by avoiding post-pasteurization contamination. It is difficult to prevent post-pasteurization contamination with the present system of milk processing. However, good plant hygiene can keep the levels  $< 5$  cfu/100 ml (144).

### ENZYMES OF PSYCHROTROPHIC BACTERIA

Although psychrotrophic bacteria cannot survive pasteurization and UHT processing, they secrete thermostable lipase, protease, and phospholipase into the milk prior to processing and can degrade milk components and ultimately affect finished product quality. Extracellular lipases secreted by psychrotrophic bacteria withstand heating at 130°C for 15 s (58). About 60% and 40% of the activity is retained following pasteurization and UHT treatment, respectively (55). The lipases produced by *P. fragi* are more thermostable and exhibit higher activity in UHT milk. Psychrotrophic bacterial counts of more than  $5 \times 10^6$  cfu/ml are required before any changes in organoleptic quality can be detected (133). A synergistic effect exists between protease, phospholipase C, and lipase, resulting in increased lipolytic spoilage of milk (23).



TABLE 1. Defects associated with growth of psychrotrophic bacteria in dairy products (22).

Product	Defect	Mechanism
Raw milk	Rancidity	Lipolytic release of free fatty acids
	Oxidized, metallic flavors	Oxidation of fatty acids to aldehydes and ketones
UHT milk	Rancidity (after 1 to 7 mon storage)	Thermostable lipases
	Gelation	Thermostable protease
Pasteurized milk	Bitty cream	Fat destabilization due to phospholipase and protease activity causing precipitation when milk is added to a hot beverage
	Gelation	Thermostable protease
	Fruity	Synthesis of esters
	Sweet curdling	Proteolysis leading to precipitation without acid production after processing
	Fouling in heat exchangers	Proteolysis leading to precipitation during processing
Pasteurized cream	Rancidity	Lipolysis
	Bitty cream	See pasteurized milk
Ice cream	Off-flavor	Lipolysis
Butter	Rancidity	Lipolysis
Milk powder	Rancidity	Lipolysis
Cheese	Taints, off-flavors	Lipolysis
	Lower yields	Proteolysis

## Phospholipase

Phospholipases are enzymes that remove fatty acids from phospholipids, and they are classified into five categories. Phospholipase A1 (EC 3.1.1.32) acts on the fatty acid in the Sn-1 position of diacylglycerol-phospholipids. Phospholipase A2 (EC 3.1.1.4) removes fatty acids from the Sn-2 position. Phospholipase B (EC 3.1.1.5) catalyzes the cleavage of the ester bonds of lysophosphatidylcholine. Phospholipase C (EC 3.1.4.3) and phospholipase D (EC 3.1.4.4) are phosphoric diester hydrolases (17). Most attention focuses on phospholipase C (PLC) as this has been shown to be produced by a variety of bacteria associated with milk spoilage and is a virulence factor in human disease (171).

*Psychrotrophic bacteria producing phospholipase.* Milk does not contain native phospholipase (52, 129), while over 50% of the bacteria isolated from milk are capable of producing phospholipase (132). Phospholipase C is produced by many genera of bacteria, including *Pseudomonas*, *Bacillus*, *Clostridium*, *Serratia*, *Acinetobacter*, *Alcaligenes*, *Citrobacter*, *Flavobacterium*, *Moraxella*, *Aeromonas*, *Chromobacterium*, and *Enterobacter* (67, 94, 129). Psychrotrophic bacteria are the principal producers of PLC in milk (23, 52).

*Factors influencing production of phospholipase.* Production of PLC is dependent on the phase of growth in *Pseudomonas*, and activity is generally detected during stationary phase of growth in *P. fluorescens* and *Pseudomonas aureofaciens* (39, 77, 151).

Nutritional factors also regulate PLC expression in *P. aeruginosa* and *Pseudomonas cepacia* PLC (104, 176). *P. fluorescens* produces maximum activity in casein-soy broth but no enzyme activity is produced in tryptone-minimal medium (77). The presence of fat in the growth medium is not a

requirement for the production of PLC (159).

**Biochemical properties of phospholipase.** Phospholipase from pseudomonads is an extracellular enzyme with molecular weight ranging between 30 and 40 kDa (33, 39, 159). *P. fluorescens* PLC hydrolyses phosphotidylcholine, phosphotidylethanolamine, and phosphotidylserine but is inactive against phosphotidylinositol (33). Similar findings are observed for a PLC purified from a *P. fluorescens* strain D that was most active against phosphotidylcholine and was least active against phosphotidylethanolamine. It showed no activity against phosphotidylinositol or sphingomyelin (77). In contrast, the enzyme studied by Doi and Nojima (39) was most active against phosphotidylethanolamine. The milk fat globule membrane (MFGM) is composed of 20.4% phospholipids, which include phosphotidylcholine (36%), phosphotidylethanolamine (27%), sphingomyelin (22%), phosphotidylinositol (11%), and phosphotidylserine (4%) (85). Considerable damage to the MFGM can be expected because the pseudomonad PLC can act upon these substrates. The differences in the substrate preferences of these enzymes indicate differences in active site configuration (39, 77).

Pseudomonad PLCs are categorized as metalloenzymes (7, 33, 39, 77, 159). It is possible that the *P. fluorescens* enzyme may be similar to a group of metallophospholipases C produced by *Clostridium novyi* (166), *Acinetobacter calcoaceticus* (100, 101), and *B. cereus* (180). It is suggested that, in these enzymes,  $Mg^{2+}$  and  $Ca^{2+}$  ions bind to the phosphate group of the phospholipid and enable recognition of the substrate by the enzyme (9, 172).

**Thermostability of phospholipase.** A number of psychrotrophic bacteria commonly found contaminating milk produce PLCs that resist both

HTST and UHT treatments (65). Fluorescent pseudomonads comprised of *Pseudomonas aureofaciens*, *P. fluorescens*, *Pseudomonas paucimobilis*, and *Pseudomonas putida* produce more thermostable PLC than do *Pseudomonas stutzeri* and *Aeromonas hydrophilia* when heated to 63°C for 30 min with 5% glycerol (24). The heat-stabilities of the different preparations of enzymes in the literature vary depending on the strain and experimental procedures (23, 33, 151, 159).

*Effect of phospholipase on milk and milk products.* Phospholipase C acts by attacking the MFGM, disrupting the integrity of the fat globule and exposing the fat to lipolytic attack by lipase (52). This is evidenced by the physical degradation of an emulsion in milk after treatment with phospholipase C (23, 67). Phospholipase increases lipolysis of raw milk at 30°C (23). The rate of initial lipolysis increases when washed cream is incubated with lipase, protease, and PLC of microbial origin. Lipolytic activity decreases after 2 h, probably due to proteolytic degradation of the lipase.

A defect in pasteurized milk known as "bitty" or broken cream is associated with bacterial phospholipases. Bitty cream is the formation of cream flecks or flakes due to agglutination of fat globules facilitated by the partial hydrolysis of the MFGM. This is brought about mainly by the action of PLC on the lecithin present in the MFGM (161). Proteases are also implicated in this defect (162). The role of PLC in enhancing lipolysis has been experimentally demonstrated by incubating a *P. fluorescens* PLC with a model emulsion consisting of butter oil emulsified with soy lecithin. When steapsin was added to this modified emulsion, an increased rate of lipolysis was observed. The activity of native milk lipase was enhanced as well (23).

*Detection of phospholipase.* Assay methods for PLC have not received

much attention. Diffusion assays using lecithin/egg yolk agar are currently used in the dairy industry (23). This method, however, requires a considerably long incubation and lacks the required sensitivity. The release of phosphoric acid due to hydrolysis of egg yolk emulsion can be measured titrimetrically (180). Alternatively, choline phosphate generation can be used as an indicator of PLC activity (151, 180).

Radio-labeled phospholipids are used to assay PLC activity (39). This method requires an additional solvent extraction step followed by thin-layer chromatography making it unsuitable for routine use in the dairy industry.

Colorimetric and fluorimetric methods based on synthetic substrates are described in a review by Stead (156). *p*-Nitrophenyl phosphoryl choline yields *p*-nitrophenol upon hydrolysis by PLC forming a yellow solution. This was the basis for an assay developed by Kurioka and Liu (94), who quantitated the released *p*-nitrophenol by measuring the absorbance at 410 nm. The generation of thiol groups from thioester analogues of lecithin is also used to determine PLC activity. A yellow complex formation by the reaction of the free thiol groups with 4,4'-dithiobispyridine was estimated spectrophotometrically. A disadvantage with this method may be the potential inhibition of enzyme activity by thiol capture agents (156).

### **Protease**

The terms proteinase (endopeptidase) and protease are often used interchangeably although proteinases are more specific in their activity than proteases, which include both endo- and exopeptidases (109). Hartley (72) classified proteases into four groups, now commonly known as: serine (EC 3.4.21), cysteine (EC 3.4.22), aspartic (EC 3.4.23), and metalloproteases (EC

3.4.24). Most of the purified proteases from psychrotrophic bacteria (predominantly those belonging to the genus *Pseudomonas*) are classified as metalloproteases because they are inhibited by EDTA (160).

***Psychrotrophic bacteria producing protease activity.*** Many psychrotrophic bacteria produce proteolytic enzymes capable of causing spoilage of milk by acting on milk proteins at low temperatures and at milk pH (Table 2). The extracellular protease produced by Gram-negative bacteria is one of the principal factors responsible for defects in milk and dairy products held at refrigeration temperatures (29, 96). *Pseudomonas*, *Acinetobacter*, *Achromobacter*, *Aeromonas*, *Enterobacter liquefaciens*, *Escherichia freundii*, *Flavobacterium*, *Xanthomonas*, *Cytophaga*, and *Proteus* are some of the proteolytic species isolated from milk (11, 130).

***Factors influencing production of protease.*** Raw milk is a unique environment for bacterial growth. Before processing, raw milk may be stored up to five days at 4 to 7°C after extensive aeration during collection. A combination of these conditions provides a complex set of factors for cell growth and enzyme production. Protease production is influenced by environmental factors such as growth temperature, aeration, growth medium, and stage of growth.

Protease production in *P. fluorescens* GTE 209 and GTE 214 was comparable at temperatures between 6°C and 21°C, but was suppressed at 2°C (140). High oxygen tension either inhibits or promotes protease production in milk depending on the strain (42, 67, 138, 139).

Protease production is also influenced by nutritional factors. In milk, proteolytic activity is first detected when cells reach high cell concentration TABLE 2. Enzyme activity of psychrotrophic Gram negative bacteria isolated from creamery silo milk (123).



Bacterial genus	Isolates with stated activity (%)		
	Lipolytic only	Proteolytic only	Lipolytic and proteolytic
<i>Pseudomonas</i> : fluorescent	5	2	71
<i>Pseudomonas</i> : non-fluorescent	32	1	11
Enterobacteriaceae, <i>Aeromonas</i> , <i>Pasteurella</i> or <i>Vibrio</i>	2	2	31
<i>Acinetobacter</i> , <i>Moraxella</i> or <i>Brucella</i>	5	9	36
<i>Flavobacterium</i>	6	6	24
<i>Chromobacterium</i>	25	6	41
<i>Alcaligenes</i>	0	0	92

toward the end of exponential growth (142). Conversely, in minimal salts medium, maximum activity occurs in mid-log phase (114), indicating nutrients also influence protease production. Caseins induce protease production in psychrotrophic *Pseudomonads* grown in a minimal salts medium while glucose, citrate, and lactose repress enzyme synthesis in minimal salts medium containing casein (130). Addition of iron to raw milk delays protease production, and iron depletion induces its production (48). Milk usually contains 0.02 mM iron, and small variations in iron levels may explain the differences in bacterial loads required for the synthesis of protease

(63). A pyoverdine negative mutant produced the same amount of protease at low or high iron concentrations, further emphasizing the role of iron in protease production (47).

The inhibition of protease synthesis by EDTA and phosphate in mineral salts medium and skim milk suggests that divalent cations may play a role in production of the active enzyme (115). Protease mRNA was synthesized irrespective of calcium content, but calcium was required for the formation of the active enzyme from the polypeptide precursor in *P. fluorescens* B52. In the absence of calcium, folding of the precursor into the active conformation was prevented (14).

**Purification of protease.** Several extracellular proteases have been purified from the culture supernatant of psychrotrophic bacteria. All the purification protocols generally consist of a pre-concentration step using methods such as ultrafiltration or freeze-drying. This is followed by two or more fractionation steps to purify the protease from the other contaminating proteins in the concentrated culture supernatant. Commonly used procedures involve ammonium sulfate fractionation and a combination of the following chromatographic procedures: anion exchange chromatography, hydrophobic interaction chromatography, and gel-filtration chromatography (54, 70, 89, 146, 179). Sexton et al. (146) used preparative SDS-PAGE in the final purification step of a protease from *Pseudomonas pseudomallei*. Enzyme yields obtained by the various isolation studies range from 0.1 to 61% (54).

Leinmüller and Christopherson (102) partially purified five proteases from a single strain of *Pseudomonas*. Fox and Stepaniak (55) purified three proteases from *Pseudomonas* AFT 21. Most of the biochemical properties of



these three enzymes were similar, including molecular weights. Stepaniak et al. (159) isolated three proteases from *P. fluorescens* P1. The major peak was studied, but two other minor peaks were not investigated. Four proteases were reported in a *P. fluorescens* AR11, but later studies on the same strain (renamed as NCDO 2085) revealed the production of a single protease with characteristics different from previous reports (4, 46). These findings imply that multiple proteases are produced in *Pseudomonas*. Several workers (70, 88, 89, 143, 146, 158) have reported production of a single protease within a strain of *Pseudomonas*. The implication of multiple enzymes for dairy product quality is unknown.

**Biochemical properties of protease.** A majority of psychrotrophic bacteria produce a monomeric protease with molecular weights ranging from 30 to 52 kDa (46, 51, 121). Four proteases are reported in *P. fluorescens* AR11 but later studies on the same strain (renamed as NCDO 2085) revealed the production of a single protease with different characteristics (4, 46).

Analysis of amino acid composition of proteases isolated from psychrotrophic bacteria revealed that they had low levels of methionine, arginine, and lysine. The isoelectric pH of the enzyme ranged between pH 5.1 and 8.8 (54).

Proteases produced by pseudomonads are zinc metalloproteases having  $Zn^{2+}$  in its active site and require  $Ca^{2+}$  for the formation and stabilization of its structure (88, 115, 143, 146). Pseudomonad proteases can be classified into two categories based on the pH optima. Neutral proteases have an optimum around pH 7.0 and alkaline proteases, between pH 8 and 9. Most *Pseudomonas* proteases, however, are active around pH 7.0 (54).

Most psychrotrophic bacterial proteases preferentially degrade caseins in the following order  $\kappa$ -casein >  $\beta$ -casein >  $\alpha$ -casein (30, 31, 46, 120). Details of the action of purified proteases from psychrotrophic bacteria on casein micelle hydrolysis are not available and are limited to unpurified preparations (30). Much of the research done with other proteases points toward preferential hydrolysis of  $\kappa$ -casein, which is reasonable since  $\kappa$ -casein is on the surface of the micelle (18, 117, 118, 131).

*Thermostability of protease.* It is estimated that 70 to 90% of raw milk samples contain psychrotrophic bacteria capable of secreting extracellular thermostable proteases that can withstand pasteurization (72°C for 15 s) and UHT processing of milk (138°C for 2 s or 149°C for 10 s) (1). Psychrotrophic bacterial proteases have decimal reduction values (D-value) of approximately 4,000 times greater than *B. stearothermophilus* spores ( $\geq 90$  s at 149°C) and are comparable to thermolysin (1, 10, 55).

*Effect of protease on milk and milk products.* The shelf-life of UHT milk is shortened by the presence of psychrotrophic bacterial protease (122). Implications of proteases on UHT milk quality are many. Proteolytic damage of caseins in raw milk lead to coagulation upon UHT treatment, formation of bitter off-flavors, gelation of milk during storage, and loss of cheese yield (1). Hydrolysis of  $\kappa$ -casein by the enzyme causes destabilization of the micelle leading to aggregation of caseins resulting in gelation and coagulation observed in UHT products (130).

High psychrotrophic bacterial counts are associated with reduced yields during cheese manufacture (8). However, there is poor correlation between psychrotrophic bacterial protease contamination and reduced Cheddar cheese yields (96, 126). Psychrotrophic bacterial counts exceeding  $10^8$  cfu/ml are

associated with reduced Cheddar cheese yields, and it is unlikely that normal psychrotrophic bacterial counts found in raw milk have an effect on cheese yield and quality (83, 96).

*Detection of protease.* Diffusion methods such as skim milk agar, gelatin agar, and elastin agar are often used for qualitative detection of protease (71, 96, 178). The requirement for extended incubation and low sensitivity of these methods restricts their use (68).

Other methods involve the assay of proteolytic products (45) that are determined using amine group specific reagents like *o*-phthaldialdehyde (25), fluorescamine (74), and 2, 4, 6-trinitrobenzenesulfonic acid (152).

Alternatively, peptides formed by hydrolysis can be precipitated by trichloroacetic acid (TCA) and quantitated by UV absorption (93), or estimated by colorimetric means using Folin-Ciocalteau reagent (50) and bicinchoninic acid (150). These methods require an additional step to separate the substrate from the products, making it cumbersome for routine use. Fluorescently labeled protein substrates like fluorescein isothiocyanate (173), 2-methoxy-2,4-diphenyl-3-2H furanone, and fluorascamine (74) are also used. The disadvantage of these is that labeling is dependent on available  $\text{NH}_2$  groups in the substrate and separation of the products from the substrate (45).

Not all of these assays are suitable for use in milk due to competition of milk proteins with the substrate. The use of immunological assays may eliminate this problem. Birkeland et al. (12) developed a plate ELISA with a sensitivity of 0.25 ng/ml. An inhibition ELISA was developed by Clements et al. (26) for the detection of *P. fluorescens* protease with a detection range between 0.24 and 7.8 ng/ml. Another ELISA technique with an improved detection limit (0.19 ng/ml) was developed by Punj and Matta (134).

Although these methods are sensitive, they take about 6 to 24 h, and the information obtained would be only of historical importance. Further, they measure total protease content, not active enzyme. A rapid protease assay is still needed despite the extensive work done to date.

### Lipase

Lipases (glycerol ester hydrolases, E.C.3.1.1.3) are hydrolases that act on the carboxyl ester bonds of acylglycerols to liberate free fatty acids and glycerol. Historically in the dairy industry, lipase is defined as "long chain fatty acid ester hydrolases" because long chain fatty acids are preferred substrates (17). However, more recently this definition has been modified to distinguish esterase and lipase activity based on the ability of a lipid-water interface to activate the enzyme. The surface area of the lipid-water interface determines the rate of lipolysis by lipase, but not esterase. Due to this requirement, lipases are defined as carboxyesterases acting on emulsified substrates (79). Esterases act on water-soluble fatty acid esters and, therefore, do not require an interface for action (110). Most previous studies have not made a distinction between an esterase and a lipase, and the two enzymes will be discussed together in this section.

Accumulation of free fatty acids  $C_4$ ,  $C_{16}$ , and  $C_{18:1}$  released as a result of hydrolysis of triacylglycerols is mainly brought on by lipase activity. Strains lacking a lipase-encoding gene (*lipA*) fail to produce these fatty acids (110).

The active site of lipases contains a catalytic triad consisting of Ser-His-Asp residues (Figure 1). This site is buried under a lid comprised of two  $\alpha$ -helices, which open in response to an oil-water interface, allowing the enzyme to attack the substrate. This "lipase-box" is conserved due to steric

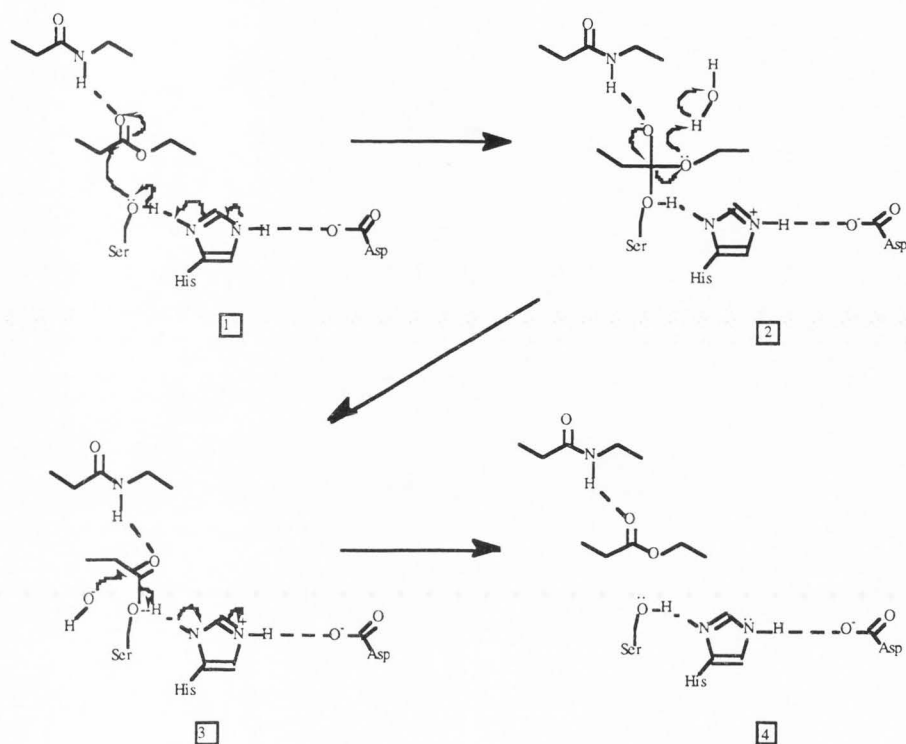


Figure 1. Mechanism of hydrolysis of an ester bond by a lipase (79).

constraints imposed by the  $\beta$ - $\epsilon$ -Ser- $\alpha$  motif (175). First, a tetrahedral intermediate (reaction 1) is formed by the nucleophilic attack of the serine side chain oxygen atom on the carbonyl carbon atom of the ester bond. The imidazole ring of histidine becomes positively charged due to protonation and is stabilized by the negative charge on the acid (reaction 2). Hydrogen bonding with amide bonds of residues in the oxyanion hole stabilizes the tetrahedral intermediate (reaction 3). The enzyme is regenerated (reaction 4) upon release of the fatty acid following a nucleophilic attack of the hydroxyl ion (79).

***Psychrotrophic bacteria producing lipase/esterase.*** Isolates of psychrotrophic bacteria that produce heat-stable lipase are characterized as aerobic, motile, Gram-negative rods belonging to the genus *Pseudomonas*

(1). The predominant lipolytic species in raw milk are fluorescent pseudomonads (148). Within fluorescent pseudomonads, *P. fragi* is more competitive than *P. fluorescens* in milk stored at low temperature (32). Other psychrotrophic bacteria associated with lipolysis of milk are *Achromobacter*, *Chromobacterium*, *Acinetobacter*, and *Alcaligenes* (35, 51).

**Factors influencing production of lipase/esterase.** Environmental conditions influence the growth and production of lipase by psychrotrophic bacteria (66). Lipase production is influenced by several factors: stage of growth, growth temperature, medium composition, aeration, and pH. These factors, coupled with the fact that milk is heat processed and stored at temperatures below the growth optimum, lead to a unique metabolic response involving alternative proteins.

In general, lipase production is maximal below the optimum growth temperature in *P. fluorescens*, *P. fragi*, *Staphylococcus carnosus*, and *Achromobacter lipolyticum* (5, 46, 55, 69, 87). Increased amounts of extracellular hydrolytic enzymes are produced at lower temperatures to compensate for the reduced diffusion and active transport of nutrients into the bacterial cell or due to decreased proteolytic deactivation of lipase at low temperatures (20, 99). Studies conducted by Merieau et al. (119) indicate that the same lipase is secreted at all temperatures in *P. fluorescens* MFO and is produced optimally at a temperature of 17.5°C. Enzyme production is suppressed at 2°C (3, 5, 125). An increase in growth temperature, however, reduces lipase production. Lipase production was reduced by 95% when the growth temperature was increased from 25 to 30°C in *P. fluorescens* (107). Maliszewska and Sroka (108) studied the influence of pH on the growth and production of extracellular enzymes in *P. fluorescens*. A pH range of 4.5 to 10



was studied, and pH 8.6 was found to be optimum for the production of lipase and protease.

Production of lipase is controlled by nutritional factors (113). Some nutrients act as inducers and others as inhibitors. Inducers include proteins, peptides, and amino acids such as glutamic acid and glutamine. Sugars, organic acids, urea, and ammonia act as repressors (46). Greater lipase activity was observed in skim milk than in whole milk when a *P. fluorescens* strain was grown at 10°C. Milkfat in whole milk may compete with the substrate resulting in an underestimation of lipase activity, or the substrate may become unavailable due to hydrophobic adsorption to milkfat (20, 24, 66, 115). A decline in the activity in skim milk was noticed after the end of the exponential phase, whereas it remained the same in whole milk. This could be due to the proteolytic action on lipase by proteases produced by *P. fluorescens*. Milkfat might have a role to play in the protection of lipase from proteolytic activity (20). Whey and isoelectric casein were shown to have a synergistic stimulatory effect on the production of lipase by *P. fluorescens* B52 (140). Iron sensitive synthesis of extracellular lipase is observed in some strains of *Pseudomonas*. Concentrations greater than 10 mM  $\text{Fe}^{3+}$  suppress lipase production (48, 116).

Fitz-Gerald and Deeth (49) studied the factors influencing lipolysis in four psychrotrophic bacteria, namely, *P. fluorescens*, *Moraxella* sp., *Acinetobacter* sp., and *P. aeruginosa*. Lipolysis was stimulated by bile salts and reduced by 33% in the presence of sodium chloride, EDTA, but metal ions such as  $\text{Fe}^{3+}$  and  $\text{Zn}^{2+}$  were inhibitory. The activity of *P. fluorescens* and *Moraxella* sp. was restored by  $\text{Ca}^{2+}$  addition.

Lipase production is dependent on oxygen tension in the culture



medium. Lipase synthesis was delayed when the culture was stationary as compared with shaken culture in whole milk at 10°C. This may be due to lower oxygen tension in static culture (20, 55). It is well documented that lack of aeration slows growth of *Pseudomonads* (16, 128). Reduced oxygen levels may be effective in controlling psychrotrophic bacteria in milk and meat products at lower temperature (91).

*Purification of lipase/esterase from psychrotrophic bacteria.* Most purification protocols for lipase purification involve a concentration step of the culture supernatant because the enzyme is extracellular. This is usually achieved by ultrafiltration, freeze-drying, and, occasionally, by acetone precipitation (76, 163). The concentrate is fractionated using iso-electric focusing, anion exchange, size exclusion, and hydrophobic interaction chromatography.

Other methods were developed to rapidly purify lipases. Wingender et al. (177) used alginate to partially purify and concentrate a *P. aeruginosa* lipase. About 95% of the enzyme was harvested from the culture medium by ethanolic precipitation with alginate. The co-precipitate was dissolved in a buffer containing detergent and separated by anion exchange chromatography. This method was applied to the purification of lipases from *Pseudomonas* sp., *Chromobacterium* sp., and *Rhizopus delemar* (78).

A single step affinity chromatography procedure was developed by Cernia et al. (21) using a functional polymer. Fatty acids of different chain lengths were esterified to polyvinyl alcohol polymers cross-linked using epichlorohydrin. A similar affinity chromatographic procedure was developed by immobilizing palmitic acid on cellulose (73). Immobilized antibodies and heparin affinity chromatography are also used to purify lipase

from mammalian sources (174).

Attempts at isolation of lipase from *Pseudomonas* indicate the production of multiple enzymes. Purification of lipase from *P. fluorescens* AFT29 by ion exchange chromatography on DEAE 32 cellulose yielded two lipase peaks. About 95% of lipase activity eluted at 0.2 M NaCl while 5% did not adsorb to the matrix and eluted before the salt gradient. These two peaks were not investigated further (42). Gel filtration of *P. fluorescens* AFT36 culture supernatant yielded three peaks with lipase activity. This was attributed to aggregation of the enzyme (55). Two lipase peaks were obtained upon gel filtration of *P. fragi* cell-free concentrate. The native molecular weight ranged from 25 to 250 kDa (99). Similar results were obtained for *Pseudomonas* sp. KWI-56 (76). Based on these data, *P. fluorescens* and *P. fragi* may produce more than one lipase.

Biochemical characterization of lipase/esterase from *Pseudomonas*. Lipase produced by bacteria belonging to the genus *Pseudomonas* exhibit diverse properties (Table 3). Lipases from *Pseudomonas* are divided into three groups based on amino acid sequence analysis (57). Group I lipases are produced by *P. aeruginosa*, *P. alcaligenes*, *P. fragi*, and *Pseudomonas* species. These have a molecular weight of about 30 kDa with 285 amino acids. Structural analysis revealed the presence of a disulfide bond involving the two cysteine residues in the molecule and the need for a "foldase" for the formation of the active protein (60). Group I lipases also form large molecular weight aggregates. This is due to the formation of micelles with lipopolysaccharide, which is the principal component of the outer membrane of Gram negative cells (59, 75, 79). Group II lipases are produced by *Pseudomonas cepacia* and *Pseudomonas glumae*. The enzyme contains about

TABLE 3. Properties of lipase from *Pseudomonas* (79).

Source of lipase	Gene sequenced	Signal sequence (aa)	Helper protein	Molecular mass	Substrate specificity	Specific features
<i>P.aeruginosa</i> PAO1	yes	yes (26)	yes	30	broad	forms high molecular weight aggregates with LPS
<i>P. aeruginosa</i> TE3285	yes	yes (26)	yes	30	n.d	no
<i>Pseudomonas</i> sp.	yes	yes (26)	n.d	30	C4-C6 F A	catalyzes lactone formation
<i>P. alcaligenes</i>	yes	yes	yes	30	Sn 1,3; C12-C18	no
<i>P. fragi</i>	yes	no	n.d	30	broad	stable at pH 9 and 50°C
<i>P. glumae</i>	yes	yes (39)	yes	33	broad	contains Ca <sup>2+</sup> binding site
<i>P. cepacia</i>	yes	yes (44)	yes	33	broad	no
<i>P.fluorescens</i> B52	yes	no	no	50.2	n.d	no
<i>P. fluorescens</i> SIKW1	yes	yes (23)	no	48	Sn 1,3; C6-C8 FA	activated by Ca <sup>2+</sup>
<i>P. putida</i>	no	n.d	n.d	45	n.d	stable at 75°C

320 amino acids with a single disulfide bridge having a molecular weight of 33 kDa (57, 82). The enzyme produced by *P. fluorescens* is classified as group III, which is clearly different from the other two groups in that it does not contain a signal sequence or cysteine residues, and does not require molecular chaperones for protein folding (44). The substrate binding region in all lipases studied thus far is highly conserved (*Pseudomonas*. sp 109, *P. fragi* 3458, *P. fragi* 12049, *P. cepacia*, *P. fluorescens* B52) (167). However, when the entire protein was compared using the protein sequence (from International Genetics Database, GenEMBL), at least four distinct lipases were found in pseudomonads (Table 4). Protein sequence similarity of the lipases varies from 4 to 70% indicating different lipases are produced by psychrotrophic pseudomonads.

**Thermostability of lipase.** Most of the psychrotrophic bacteria present in milk, particularly *P. fluorescens* produce extremely heat-stable extracellular lipase. Early studies documented this remarkable thermal stability on a broad level. Later studies have gathered more precise data (24, 55, 112, 145). Driessen and Stadhouders (41) were the first to report heat-resistant lipase production in *P. fluorescens* 22F, with a D-value of 4.8 min at 150°C. Other workers (2, 6, 15, 122) have confirmed the heat stability of the enzyme at both pasteurization and UHT temperatures. Makhzoum et al. (107) used fluorescence spectrophotometry to study the conformational stability of a *P. fluorescens* lipase by determining thermodynamic parameters such as free energy ( $\Delta G$ ), enthalpy ( $\Delta H$ ), entropy ( $\Delta S$ ), and heat capacity ( $\Delta C_p$ ) for the unfolding reaction. They were determined to be 9.5 kJ/mol, 151 kJ/mol, 475 J/mol/K, and 4.06 kJ/mol/K, respectively. The unfolding reaction was shown to be reversible. Of the 20 lipases produced by

TABLE 4. Comparison of identity of different lipase protein sequences.<sup>a</sup>

Comparison	Sequence identity (%)	Comments
LipA between species ( <i>P. cepacia</i> vs. <i>P. aeruginosa</i> )	22	fairly unrelated enzymes
LipA vs. LipL ( <i>P. cepacia</i> vs. <i>P. fragi</i> )	45	LipA and LipL from these species are similar
LipL vs. <i>P. fluorescens</i>	5	unrelated
LipA vs. <i>P. fluorescens</i>	4	unrelated
<i>P. fluorescens</i> genomic vs. <i>P. fluorescens</i> triacylglycerol lipase	70	highly related proteins
<i>P. fluorescens</i> genomic, <i>P.</i> <i>fluorescens</i> triacylglycerol lipase vs. LipA	4	unrelated
<i>P. fluorescens</i> genomic, <i>P.</i> <i>fluorescens</i> triacylglycerol lipase vs. LipL	15	unrelated

<sup>a</sup> Proteins used in this comparison were deduced from the gene sequence deposited in GenBank.

psychrotrophic bacteria that were studied, 44% showed activation on heating. This activation may be due to the adsorption of lipase to cream, bringing the enzyme and its substrate in close proximity or due to a conformational change to a more stable form (92, 168). Prolonged heating, however, showed a decline in activity (10, 49). Biphasic inactivation of the enzyme observed in the temperature range of 40 to 110°C also indicates the production of more than one enzyme or that the enzyme assumes different conformations (41,

165). Extent of aggregation could also explain this kind of inactivation kinetics (106). Comparison of heat stabilities reported in different studies is difficult since residual activity depends on methodology, heating media used, degree of purification of the enzyme, and the strains employed (148).

*Effect of lipase on milk and milk products.* Lipases release FFA into milk due to hydrolysis of triacylglycerides. Pseudomonads are responsible for a large proportion of lipolytic spoilage in processed milk (84). The phospholipid bilayer membrane with embedded proteins (86) surrounds milk triacylglycerides. The MFGM is primarily composed of phospholipids and maintains the globular structure of milk fat rendering it resistant to lipolysis (162). If the membrane is damaged, the interior triacylglycerides are exposed to hydrolytic enzymes, such as lipase. Mechanical processing steps, such as homogenization, high speed pumping, and agitation disrupt the MFGM. Additionally, the synergistic action of protease and phospholipase damage the MFGM by hydrolyzing the proteins and phospholipids in the membrane (23). The triacylglycerides, therefore, become more susceptible to the action of lipase (86).

Lipase adsorbs onto the surface of the MFGM and is, therefore, concentrated in butter and cheese and, unlike protease, is not lost in the whey portion of fermented milk products. As a result of this property, lipase poses a problem in long shelf-life dairy products including cheese, butter, and UHT fluid milk (37, 40). Consequently, the growth and production of heat-stable enzymes by psychrotrophic bacteria pose a major problem in products intended for distant markets because they remain active after pasteurization and UHT treatment of fluid milk (29).

Lipases bring about the hydrolysis of milk triacylglycerides and generate



free fatty acids. A positional preference for Sn-3 position by most bacterial lipases results in the liberation of butyric and caproic acids as these fatty acids are commonly found occupying the third position on the glycerol backbone. Low levels of lipolysis result in decreased product acceptance because these fatty acids are volatile and are associated with unpleasant flavor (36). Fatty acids between the chain lengths of  $C_4$  and  $C_8$  are associated with rancid flavors and those between  $C_{10}$  and  $C_{12}$  with unclean and soapy flavors. Fatty acids of longer chain lengths from  $C_{14}$  to  $C_{18}$  contribute minimally to flavor changes. The released free fatty acids may further undergo oxidation to aldehydes and ketones resulting in cardboardy, oxidized, and metallic flavor (155). Additional flavor defects result due to the formation of volatile esters from the released free fatty acids by the involvement of another group of enzymes called esterases. *P. fragi* is responsible for the development of a fruity odor in spoiled milk, cheeses, and meats (22, 29, 34, 64, 135).

Enzyme action also leads to some technological problems in addition to deterioration of sensory attributes and product acceptance. Lipolysis of milk fat results in decreased processing ability of milk in addition to causing flavor defects. Some of these problems are poor churning during butter-making, decreased foaming in making cappuccino coffees, inhibition of starter growth by free fatty acids during cheese-making, and weak or delayed rennet clotting (19, 28, 92, 169).

**Detection of lipase.** Several methods exist for the detection of lipase activity. It is important to have a rapid test as the information could be used to direct milk for processing into different products depending on the quality. Diffusion assays are the simplest methods used to detect lipase activity where a gel (usually of agar containing an indicator compound) is



cast. Wells are cut in the agar and the supernatant is filled in the well. A zone of clearing or color change of the indicator is measured to determine the enzyme activity (123). Some diffusion assays commonly used are tributyrin agar and Sierra agar (111).

Various other methods are used to detect lipase activity, which are based on continuous (pH-stat technique) or discontinuous titration of the released fatty acid (38). These methods are much too slow and lack the sensitivity required to test fresh milk to be used routinely in the dairy industry. Lipase activity can be monitored using radioactivity, bioluminescence, chromogenic substrates, fluorogenic substrates, and ELISA (111). Characterization of lipase specificity of purified enzymes is done using substrates such as tributyrin, olive oil, milk fat, and methylbutyrate, but they provide limited information regarding specificity for chain length or position unless linked to a more sophisticated detection system not readily available in a factory laboratory (55, 136).

Roy (141) and Stead (154) developed a rapid fluorimetric assay in response to the need. This is based on the hydrolysis of 4-methylumbelliferyl oleate. The usefulness of the method is limited by its requirement for bile salts, which have varying effects on the activity of psychrotrophic bacterial lipase (49). McKellar (111) developed a colorimetric assay that measures the development of a purple color due to the release of  $\beta$ -naphthol from the colorless substrate  $\beta$ -naphthyl caprylate. This method also suffers from being too cumbersome for routine use in the dairy industry as it requires an additional quenching step followed by centrifugation and labeling with a second indicator dye. Richardson et al. (137) used reflectance colorimetry to monitor the reduction in pH due to release of free fatty acids from tributyrin.

This method, however, did not prove useful in the dairy industry, as the detection limit was too high. To develop a rapid assay suitable for use in milk, Blake et al. (13) used reflectance colorimetry to measure the release of *p*-nitrophenol from *p*-nitrophenol caprylate. This method is rapid and can be used in turbid solutions such as milk.

### **SOME APPLICATIONS OF PSYCHROTROPIC BACTERIAL ENZYMES**

The estimated sales of enzymes in 1995 was \$1 billion. This figure is projected to increase to \$2 billion by the year 2005. Enzymes of microbial origin account for about 90% of all enzymes being used industrially. Approximately 75% of the enzymes employed by the industry are hydrolytic in nature and are used for depolymerization of complex substances found in nature (62).

Proteases account for about 40% of the enzyme sales. Some of the areas of applications of proteases are listed in Table 5. These are chiefly used in the dairy and detergent industry (62). Lipases are also found to be industrially useful due to their ability to preferentially hydrolyze long/short or saturated/unsaturated fatty acid residues (105). Lipases are able to remain active in organic solvents where they can catalyze various synthetic reactions. The versatility of these enzymes is being exploited in the industry to produce a variety of compounds (59). Some of the applications of these enzymes are summarized in Tables 5 and 6.

Technological advances for the acceleration of cheese ripening, low-fat cheese development, and production of altered milk components by using recombinant DNA techniques have brought on a need to avoid the potential

TABLE 5. Some applications of lipases (59).

Application	Comments
<b>Food industry</b>	
Modification of triacylglycerols	Used to upgrade inexpensive oils by regioselective replacement of fatty acyl residues e.g., cocoa butter equivalents are made from palm oil and stearic acid for use in the confectionary industry
Hydrolysis of triacylglycerols	Used to release free fatty acids for use as surfactants. The enzymatic process reduces energy requirements and prevents product deterioration as compared to chemical methods
Ripening and fermentation	Development of distinctive flavor of the product e.g., by selective hydrolysis of milk lipids during cheese manufacture
Ester synthesis	Used to produce natural flavor esters as alternatives to synthetic ones
<b>Speciality chemicals</b>	
Synthesis of novel chemicals and resolution of racemic mixtures	Stereoselective properties of lipases are used to produce optically pure chemicals (e.g., $\beta$ -blockers, insecticides, and herbicides)
<b>Miscellaneous</b>	
Detergent formulations	Used along with other enzymes like proteases or amylases
Paper industry	Used to reduce pitch build-up on machinery during paper manufacture
Biosurfactants	Used to synthesise carbohydrate esters that are used as alternatives to petroleum-based detergents
Environmental management	Used to unclog drains in fast-food outlets

TABLE 6. Applications of some industrially useful proteinases (61).

Enzyme/source	Type	Applications
Rennet/calf, microbial	Aspartic	milk coagulant
Chymotrypsin	Serine	de-allergizing
Pepsin	Aspartic	general hydrolysis
Trypsin	Serine	tanning leather
Alkaline protease/ <i>Bacillus</i> spp.	Serine	detergents
Neutral proteinase/ <i>B. subtilis</i>	Metallo	general hydrolysis, acceleration of Cheddar cheese ripening
Ficin/fig latex	Cysteine	general hydrolysis
Bromelain/pineapple stem and fruit	Cysteine	general hydrolysis, meat tenderization
Papain/papaya fruit	Cysteine and mixed	general hydrolysis, meat tenderization

defect of bitterness in cheese (51, 53, 103, 124). The use of enzymes produced by *Pseudomonas* in reducing bitterness and product improvement has been explored. A neutral proteinase from *Bacillus subtilis* enhances flavor of Cheddar cheese at low levels of addition but causes bitterness at higher levels (98). Similarly a preparation containing proteinase-peptidase from *Pseudomonas* was added to the curd during salting of Cheddar cheese. Results indicate that low levels of added enzyme accelerate and enhance ripening while large amounts cause bitterness (95). These studies suggest that pseudomonad proteases can be used to accelerate ripening in cheese when

used in moderate amounts. *Pseudomonas* can grow easily on inexpensive minimal media and addition of either washed cells or crude culture concentrates will not affect the cost of cheese-making (56).

Niland and Fox (127) used washed cells of *Pseudomonas tolaasii* to enhance ripening of Cheddar cheese. Their findings indicate that the addition of cells improved flavor and no off-flavors were detected. No increased proteolysis as determined by PAGE was detected.

### CONCLUSIONS

*Pseudomonads* grow in raw milk at refrigeration temperatures and dominate the raw milk microflora. They secrete extracellular enzymes such as phospholipase, lipase, and protease during their growth. Most of these enzymes are thermostable to varying extents and threaten the quality of long shelf-life dairy products. Production of these enzymes is dependent on various growth factors. Isolation of enzymes suggests that more than one enzyme may be produced, but has not been proven conclusively. Production of multiple enzymes in response to different conditions to which milk is exposed during processing further intensifies the problem by making their detection and control difficult. This study explores the possibility of the existence of multiple phospholipase, lipase, and protease in some raw milk isolates of *Pseudomonas* by studying the influence of growth conditions on production and thermostability of these enzymes. Production of multiple enzymes was also investigated using immunological techniques and isolation and characterization of enzymes.



## HYPOTHESIS AND OBJECTIVES

Psychrotrophic pseudomonads produce a single phospholipase, protease, and lipase that are heat-stable and lead to spoilage in dairy products. The following objectives were fulfilled in order to test this hypothesis.

1. Screen 17 strains of *Pseudomonas* for total and heat-stable lipase, protease, and phospholipase activity.
2. Determine if incubation time has an influence on total and heat-stable phospholipase, lipase, and protease activity.
3. Determine if medium has an influence on total and heat-stable phospholipase, protease, and lipase activity.
4. Isolate and characterize lipase(s) from *Pseudomonas fluorescens* RO98.
5. Isolate and characterize protease(s) from *Pseudomonas fluorescens* RO98.
6. Determine ability of protease to hydrolyze hydrophobic peptides found in Cheddar cheese.
7. Investigate an immunomagnetic method for protease assay.

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### CHAPTER III

## INFLUENCE OF GROWTH CONDITIONS ON PHOSPHOLIPASE PRODUCTION IN *PSEUDOMONAS*

### ABSTRACT

Many psychrotrophic bacteria contaminating raw milk produce phospholipase that withstands pasteurization and UHT treatments. This enzyme acts on the milk fat globule membrane and exposes triacylglycerides to the action of lipase. Phospholipase production by various raw milk and environmental isolates of *Pseudomonas* was investigated. The isolates were cultured aerobically at 8°C in nutrient broth, McKellar's minimal salts medium, Chrisope's medium, and skim milk. Each strain was studied for production of phospholipase during a 50-h incubation. Enzyme production varied significantly ( $P < 0.001$ ) with strain and growth medium. Strains varied significantly ( $P < 0.001$ ) in their enzyme production in each medium and during the incubation time as well. Strain, stage of growth, and the growth medium significantly influenced ( $P < 0.001$  to 0.0924) heat stability of the enzyme activity. Phospholipase production varied with environmental conditions. Thermoprocessing reduced the activity, but did not eliminate it in skim milk.

### INTRODUCTION

Although most spoilage organisms associated with milk produce phospholipase, it is the least studied extracellular enzyme with regard to spoilage of dairy products (10, 13). Phospholipases (EC 3.1.4.3) (Phosphatidylcholine choline-phosphohydrolases) are a group of enzymes



that act on phospholipids. Most bacterial phospholipases hydrolyze phospholipids to diglycerides and substituted phosphoric acid and are classified as phospholipase C (PLC) (4).

Phospholipase C disrupts the integrity of the milk fat globule membrane (MFGM) and exposes the fat to lipolytic attack by lipase (1, 5, 7). This results in the physical degradation of the emulsion in milk (9, 23). Damage to the MFGM is important in long shelf-life products such as whipping cream and ice cream where functional and sensory attributes are affected (2, 19). Phospholipase isolated from *Pseudomonas fluorescens* increases lipolysis of raw milk at 30°C (10). The rate of initial lipolysis increased in washed cream incubated with lipase in the presence of both protease and PLC of microbial origin at pH 6.6 and 37°C (1). Additionally, phospholipase C activity is responsible for sweet curdling, bitterness, and feathering in milk (2, 25).

Milk does not contain native phospholipase (24, 25). Over 50% of the bacteria isolated from milk are capable of producing phospholipase. Psychrotrophic bacteria are the principal producers of PLC in milk (25). Phospholipase C is produced by many genera of bacteria, including *Pseudomonas*, *Bacillus*, *Clostridium*, *Enterobacter*, *Serratia*, *Acinetobacter*, *Alcaligenes*, *Citrobacter*, *Flavobacterium*, *Moraxella*, *Aeromonas*, *Chromobacterium*, *Bacillus*, and *Enterobacter* (25, 26).

The enzyme activity is extracellular and is produced during late exponential and stationary phases of growth (5). Reports regarding heat stability of the enzyme vary. Heat resistance of the enzyme in batch pasteurization conditions (62.5°C for 30 min) is not established.

Growth medium influences PLC activity of *Pseudomonas aeruginosa*

and *Pseudomonas cepacia* (17, 20, 32). Influence of growth medium on raw milk-contaminating pseudomonads is lacking. Ivanov et al. (15) found no activity in tryptone mineral medium, while Stepaniak et al. (30) found no such differences in PLC activity due to medium in *P. fluorescens*. This study was undertaken to determine the influence of growth medium on PLC activity and heat-stability of the enzyme.

## MATERIALS AND METHODS

### Strains and Media

Seventeen isolates of *Pseudomonas* were used in this study (Table 7). Of the 17 strains, nine strains were raw milk isolates (9, 11). Working cultures were prepared from frozen culture stocks stored at  $-70^{\circ}\text{C}$  in reconstituted nonfat dry milk containing 20% glycerol. An inoculum was freshly prepared from a 24-h culture (0.2 optical density) by harvesting the cells with centrifugation at  $6000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The inoculum was washed three times with sterile saline and resuspended in sterile saline to an  $\text{OD}_{620}$  of 0.2. This was used to inoculate each sterile medium. The media used in this study were nutrient broth (Difco Laboratories, Detroit, MI), McKellar's minimal medium with lipase inducer (21), UHT skim milk (Gossner Foods, Logan, UT), and Chrisope's medium (5). Composition of the media is listed in Table 33 (Appendix). Inoculated media were incubated at  $10 \pm 2^{\circ}\text{C}$  with shaking at 200 rpm in a 500-ml flask.

Lipase inducer for McKellar's medium was prepared from UHT skim milk by acidification with 1 N HCl to pH 4.6 to precipitate the caseins followed by centrifugation ( $7000 \times g$  for 10 min at  $4^{\circ}\text{C}$ ), and filtration through a Whatman #1 filter paper (Whatman International Ltd., Maidstone, UK). The

TABLE 7. Strains used to study phospholipase.

Organism	Strain name	Code	Source/ reference	Comments
<i>P. fluorescens</i>	38	1	9	milk spoilage
<i>P. fluorescens</i>	CHA96	2	18	GacA <sup>-</sup> /pME3066
<i>P. fluorescens</i>	CHA0	3	18	GacA <sup>+</sup> , wildtype
<i>P. fluorescens</i>	CHA89	4	18	GacA <sup>-</sup> , mutant
<i>P. fluorescens</i>	3	8	9	milk isolate
<i>P. fluorescens</i>	RO98	9	11	raw milk isolate
<i>P. fluorescens</i>	B52	11	28	raw milk isolate
<i>P. fluorescens</i>	RO13	12	11	raw milk isolate
<i>P. fluorescens</i>	AFT29	13	12	
<i>P. fluorescens</i>	AFT36	14	12	
<i>P. fluorescens</i>	948	15	ATCC	
<i>P. fluorescens</i>	31732	16	ATCC	
<i>P. fluorescens</i>	LS107d2	17	16	
<i>P. fragi</i>	71	5	9	milk spoilage
<i>P. putida</i>	345	7	9	milk spoilage
<i>Pseudomonas</i> sp.	113	6	9	milk spoilage
<i>P. xanthophilia</i>	RO28	10	11	raw milk isolate

filtered whey was neutralized to pH 7.0 using 1N HCl and filter-sterilized through a 0.22- $\mu$ m syringe filter (Gelman Sciences Inc., Ann Arbor, MI) and stored at -70°C until required. This was added (1% v/v) to sterile McKellar's minimal medium.

### Sampling

Samples (10 ml) were drawn from each medium at 24 and 50 h and divided into two subsamples. One half was heat treated at  $62.5 \pm 0.5^\circ \text{C}$  for 30 min in a water bath and the other half was not given a heat treatment. These two subsamples were subsequently referred to as heated and not-heated, respectively. The measurements were done in duplicate.

### Total Plate Counts

Total plate counts were determined at both 24 and 50 h before heat treatment of the culture using the standard spread plate method (27).

### Phospholipase Assay

A lecithin agar diffusion assay was used to determine phospholipase activity, which measures hydrolysis of phosphotidyl choline (6). Plates containing 20 ml of lecithin agar were used to make four wells of 4 mm diameter. The plates were incubated overnight at 30°C to check sterility following which contaminated plates were discarded. Heated and not-heated culture supernatants were dispensed into each well (30 µl) and covered with the lid. The sides of the plate were sealed with parafilm and incubated upright at 30°C for 24 h. Zones of opacity formed due to hydrolysis of phosphotidyl choline were measured in millimeters. Two measurements taken at right angles to each other were averaged. A negative control consisting of sterile medium was subtracted from the sample value. Plate counts were used to calculate activity obtained per cell (Eq 1). Enzyme activity was expressed as activity units/cell (U), which was defined as millimeters opacity zone obtained per cell in 24 h.

$$\text{PLC activity (U)} = (\text{Average zone (mm)} / \text{Average plate count}) \times 10^8 \quad [\text{Eq. 1}]$$

### Statistical Analyses

Data were analyzed by analyses of variance in a  $17 \times 4 \times 2 \times 2$  (Eq. 2) factorial design using the Minitab statistical software (Release 9.1, Minitab, Inc., State College, PA). The three-way and four-way interaction mean squares were pooled to obtain the error term since no replication was done

(pers. comm., Dr. D. Sisson, July, 1998) (Eq. 3). Least square difference (22) tests were used to calculate the differences between means ( $\alpha < 0.05$ ).

$$Y_{ijklm} = \mu + S_i + M_j + H_l + Sm_{ij} + T_k + St_{ik} + Mt_{jk} + Th_{kl} + SH_{il} + MH_{jl} + \text{Error} \quad [\text{Eq. 2}]$$

$$\text{Error} = SMT_{ijk} + SMH_{ijl} + STH_{ikl} + MTH_{jkl} + SMTH_{ijkl} + d_{ijklm} \quad [\text{Eq. 3}]$$

S = strain; M = medium; T = incubation time;

H = heat treatment; Y = phospholipase activity, d = duplication

## RESULTS

### Influence of Medium, Strain, Incubation Time, and Heat Treatment

The main effects of medium, strain, incubation time, and heat treatment significantly influenced ( $P < 0.001$ ) PLC activity (Table 8). Growth in Chrisope's medium significantly decreased the PLC activity compared to growth in the other three media (Figure 2A).

Strains produced significantly different ( $P < 0.001$ ) amounts of PLC activity (Table 8), which varied from 0 to 7.8 U (Figure 2B). Little or no enzyme activity was observed in *Pseudomonas* sp. 113 (code 6), *P. putida* 345 (code 7), and *P. xanthophilia* RO28 (code 10). *P. fragi* 71 (code 5) produced the most activity (7.8 U). The fluorescent pseudomonads (codes 1 to 5, 8 to 9, and 11 to 17) produced higher amounts of activity compared to non-fluorescent pseudomonads (Codes 6, 7, and 10).

Increasing incubation time significantly increased ( $P < 0.001$ ) PLC activity (Table 8 and Figure 2C). Enzyme activity was detected at 24 h of

TABLE 8. ANOVA for phospholipase production.

Treatment	df	P-value
Medium (M)	3	<0.001
Strain (S)	16	<0.001
Incubation time (I)	1	<0.001
Treatment (T)	1	<0.001
M x S	48	<0.001
M x I	3	0.7892
M x T	3	0.0924
S x I	16	<0.001
S x T	16	<0.001
I x T	1	0.0196
Error <sup>a</sup>	163	—

<sup>a</sup>Error term was summed over duplication, three-way and four-way interactions.

growth and increased 110% at 50 h (Figure 2C). Once the enzyme was formed, pasteurizing the culture supernatant significantly decreased activity ( $P < 0.001$ ) (Table 8). After pasteurization, about 47% of the activity was retained independent of the strain (Figure 2D).

#### Interactions and PLC Activity

Medium did not significantly influence heat stability of the enzyme ( $P = 0.0924$ , Table 8, Figure 30B, Appendix), nor did incubation time ( $P = 0.7892$ , Table 8, Figure 30A, Appendix). PLC activity was significantly influenced ( $P < 0.001$ ) by the interaction between medium and strain (Table 8).



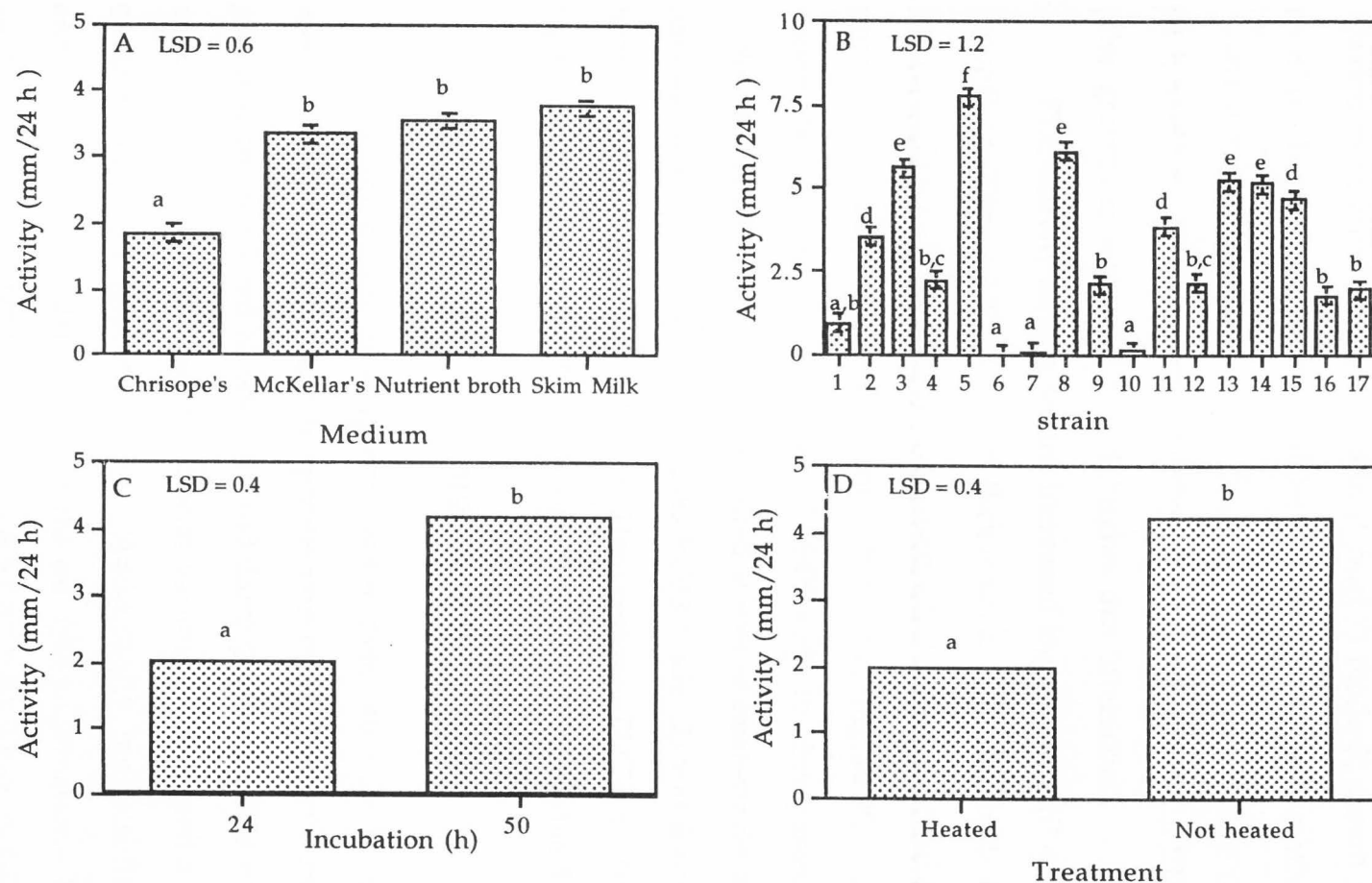


Figure 2. Influence of growth conditions on phospholipase activity. Panel A shows the influence of growth medium, Panel B shows the influence of strain, Panel C shows the influence of incubation time, and Panel D shows the influence of heat treatment. Activity has been averaged over the other factors. Strain numbers correspond to the strain names in table 7. Error bars represent standard error of the mean. In panels C and D the error bars do not show due to the small magnitude. Bars with same letters are not significantly different.

*Pseudomonas* sp. 113 (code 6), *P. putida* (code 7), and *P. xanthophilia* RO28 (code 10) had little or no activity in the four media tested (Figure 3A). Maximum activity was observed in *P. fragi* 71 (code 5) grown in McKellar's medium, but contained lower activity when grown in the other media. Alternatively, PLC activity in *P. fluorescens* CHA0 (code 3) increased with each medium. The difference between these two strains in their PLC activity after growth in each medium highlights this interaction.

PLC activity among strains increased significantly ( $P < 0.001$ ) with incubation (Figure 3B), except in *Pseudomonas* spp. 113 (code 6) and *P. putida* 345 (code 7) where no activity was observed. Heat-stability of PLC activity produced by each varied significantly ( $P < 0.001$ ) (Figure 3C). Each supernatant retained an average of 34.94% activity after pasteurization. *P. fluorescens* CHA96 (code 2) exhibited the most heat-stable activity (79.1% residual activity) while, *P. fluorescens* RO13 (code 12) was found to be least heat-stable (7.7% residual activity). Heat resistant PLC activity also significantly increased with incubation time ( $P = 0.019$ , Table 8, Figure 3D).

## DISCUSSION

The aim of this investigation was to determine the influence of medium and strain variation for production of PLC activity in pseudomonads related to raw milk and dairy products. This was done by conducting a factorial experiment with various growth media, strain combinations, and incubation times. Additionally, heat stability of the enzyme activity was determined in the cell free supernatant. The data were analyzed with an ANOVA using the sum of duplication three-way and four-way interactions as the error term. This is a conservative approach to the

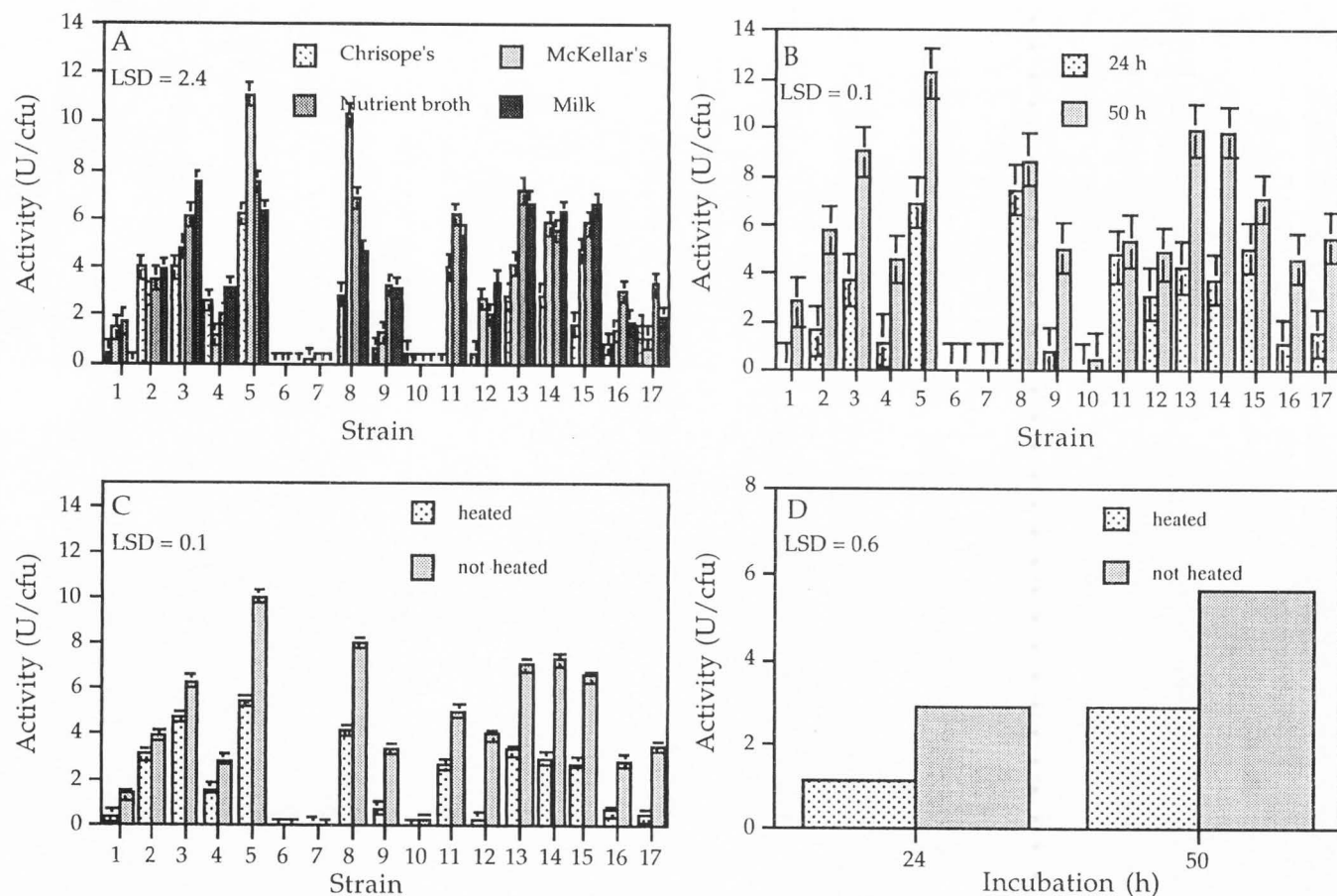


Figure 3. Influence of growth conditions on phospholipase activity. Panel A shows the influence of strain x medium, panel B shows the influence of strain x incubation time, and panel C shows the influence of strain x heat-treatment, panel D shows the influence of incubation time x heat treatment ( $62.5 \pm 0.5^\circ\text{C}$ ). Activity has been averaged over the other factors. Strain numbers correspond to the strain names in Table 7. Error bars represent standard error of mean.

analysis (pers. comm., Dr. D Sisson, July, 1998) when replication was not conducted. With this in mind, the conclusions drawn will under estimate the influence of each factor, compared to using an error term based on replication rather than duplication.

Medium, strain, incubation time, and pasteurization significantly influenced ( $P < 0.001$ ) PLC activity (Table 8). Four of six two-way interactions significantly influenced ( $P < 0.001$  to  $0.02$ ) PLC activity as well. Growth in Chrisope's medium produced the least amount of PLC activity, despite being designed to maximize this activity (5). This medium is a combination of peptone and yeast extract as the nitrogen source and was optimal for PLC production in *P. fluorescens* 178. It is interesting to note that a medium developed for optimum PLC production in one strain was not optimum for others used in this investigation. Conversely, growth in skim milk resulted in the maximum PLC activity (Figure 2A). Milk is therefore an adequate medium for PLC production. Raw milk and dairy products will contain PLC activity assuming they are contaminated with pseudomonads, which is likely (8, 13, 14, 19).

With these findings in mind and the fact that milk composition varies with diet and stage of lactation (24), it is reasonable to suspect that PLC activity in milk will vary due to factors beyond the scope of this work. For example, it would be interesting to determine the relationship of PLC activity and milk composition in systems that have continuous milk and in those that have a seasonal milk supply (such as New Zealand and Australia). No studies in the literature were found that made this connection.

All but three strains produced PLC activity (Figure 2B) in the growth media used (Figure 3A). The amount produced by each strain varied

significantly ( $P < 0.001$ ) depending on the medium used for growth ( $P < 0.001$ ). Strain *P. fragi* 71 (code 5) produced the most activity irrespective of medium and growth time, while strain *Pseudomonas* sp. 113, *P. putida* 345, and *P. xanthophilia* RO28 (codes 6, 7, and 10, respectively) produced little or no activity in any condition (Figure 2B). Milk was one of the best media for PLC production for all but *P. fluorescens* 38, *Pseudomonas* sp. 113, *P. putida* 345, and *P. xanthophilia* RO28 (codes 1, 6, 7, and 10) (Figure 3A). These findings indicate that, irrespective of what strain contaminates milk, PLC activity will be produced and available to cause spoilage. Hence, it is important to minimize the amount of PLC activity left in finished dairy products.

The influence of incubation time and pasteurization was investigated as mechanisms to reduce the amount of PLC activity in raw milk that enters the processing plant. Activity increased significantly ( $P < 0.001$ ) during incubation to 50 h (Figure 2C), but also varied significantly ( $P < 0.001$ ) by strain (Figure 3B). Longer incubation produced more PLC activity, and the most activity was present in *P. fragi* 71 (code 5) after 50 h of incubation. Enzyme production at 24 h and 50 h of incubation corresponded to the late exponential and stationary phase of growth, respectively (3). PLC activity is noted to be a stationary phase metabolite in *P. aureofaciens* (29), but the present investigation observed PLC activity earlier than stationary phase suggesting that their expression is differently regulated. This interaction indicates that the longer the raw milk is stored the more PLC activity it will contain, but the absolute amount of activity present will depend on the milk composition and the strains growing in milk. If these organisms produce PLC activity in exponential phase, reduction of cells numbers may be one of the

only mechanisms for control of this enzyme. Additionally, long shelf products contaminated with pseudomonads (via post-processing contamination), which is common (9), may contain elevated levels of PLC activity that degrade the product and change the functionality (2, 7, 23).

Pasteurization significantly reduced ( $P < 0.001$ ) PLC activity (Figure 2D). Heat processing to eliminate the enzyme was unsuccessful (Figure 2D) irrespective of the raw milk holding time (Figure 3D). Therefore, raw milk should be protected from pseudomonad contamination before and after processing to reduce the level of PLC in dairy products. Traditional temperature processing strategies will not eliminate, but will reduce the amount of enzyme in milk after processing (15, 31). Additionally, the heat treatment varied significantly with strain ( $P < 0.001$ ) (Figure 3C) and the length of incubation ( $P < 0.02$ ) (Figure 3D). A higher proportion of activity was retained following heat treatment of the 50-h culture supernatant compared to the 24-h supernatant. This may be due to the effect of increased protein concentration in the supernatant, to the production of some other protective metabolite, or to an additional PLC enzyme with different characteristics from the one produced at 24 h.

## CONCLUSIONS

Phospholipase activity in pseudomonads varied with growth medium and incubation time. Heat stability of the enzyme also varied with strain, medium, incubation time, and heat treatment. In all cases increased incubation time resulted in higher and more heat-stable PLC activity than shorter incubation times. Growth in skim milk resulted in significantly higher PLC activity compared to Chrisope's medium. Hence, it is likely that



raw milk will contain PLC activity at different amounts depending on the strains present and the time held before processing based on the observations of this study. Elimination of this enzyme was not accomplished by heat processing alone; however, a reduction was observed after pasteurization. Therefore, it will also be active in the finished product. Strategies to reduce this enzyme in milk and dairy products include techniques to prevent pseudomonad contamination and ensure short storage before processing. Milk containing high PLC activity may not be suitable for long shelf-life products, such as milk powder or UHT milk, as a result of subsequent defects that will appear during storage.

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CHAPTER IV  
INFLUENCE OF GROWTH CONDITIONS ON PROTEASE  
PRODUCTION IN *PSEUDOMONAS*

ABSTRACT

Protease production in pseudomonads was investigated in various growth conditions. Seventeen raw milk and environmental isolates were grown aerobically in nutrient broth, McKellar's minimal salts medium, Chrisope's medium, and skim milk at  $10\pm^{\circ}\text{C}$ . Each strain was studied for protease production during a 50-h incubation period. Protease activity varied significantly ( $P < 0.001$ ) with medium, strain, heat treatment, and incubation time. Strain and growth duration significantly influenced ( $P < 0.001$ ) heat stability of the enzyme activity. Protease activity varied with environmental conditions. Thermoprocessing reduced the activity, but did not eliminate it in skim milk.

INTRODUCTION

Psychrotrophic bacteria are of concern in the dairy industry because they grow at refrigeration temperatures and produce enzymes such as lipase, protease, and phospholipase that are not only heat stable, but also active at refrigeration temperatures (31). Proteases assume greater significance than the other two enzymes due to their remarkable heat stability, contribution to spoilage of milk during storage, off-odor/flavor production, reduction of cheese yields, and gelation of UHT milk (2, 15). Consequently, consumer acceptance and shelf-life of milk are reduced (7, 11). Limiting raw milk storage time is important in reducing heat-stable enzyme

activity in finished dairy products.

Most reports indicate that maximal activity occurs during late exponential and early stationary phase (20, 25, 33). In milk, proteolytic activity is first detected when cells reach high concentrations toward the end of exponential growth (29). Conversely, in minimal salts medium, maximum activity occurs in mid-exponential phase (21). However, little is known about the influence of media and culture conditions on properties of protease. Knowledge regarding these factors will lead to a better understanding of the problem with psychrotrophs and may help control enzyme activity in dairy products (11, 12).

Leinmüller and Christopherson (18), who partially purified five proteases from a single strain of *Pseudomonas*, suggested production of multiple enzymes. Stepaniak et al. (32) isolated three proteases from *Pseudomonas fluorescens* P1. The major peak was studied but two other minor peaks were not investigated. Most of the biochemical properties of these three enzymes were similar, including molecular weight. Hence, isolation of the same enzyme cannot be ruled out.

The present study examined the influence of growth medium and incubation time on the production and heat stability of protease by various *Pseudomonas* strains of dairy origin.

## MATERIALS AND METHODS

### Strains and Media

Seventeen isolates of *Pseudomonas* were used in this study (Table 9). Of the 17 strains, nine strains were raw milk isolates (8, 9). Working cultures were prepared from frozen culture stocks stored at  $-70^{\circ}\text{C}$  in reconstituted

TABLE 9. Strains used to study protease.

Organism	Strain name	Code	Source/ reference	Comments
<i>P. fluorescens</i>	38	1	8	milk spoilage
<i>P. fluorescens</i>	CHA96	2	17	GacA <sup>-</sup> /pME3066
<i>P. fluorescens</i>	CHA0	3	17	GacA <sup>+</sup> , wildtype
<i>P. fluorescens</i>	CHA89	4	17	GacA <sup>-</sup> , mutant
<i>P. fluorescens</i>	3	8	8	milk isolate
<i>P. fluorescens</i>	RO98	9	9	raw milk isolate
<i>P. fluorescens</i>	B52	11	28	raw milk isolate
<i>P. fluorescens</i>	RO13	12	9	raw milk isolate
<i>P. fluorescens</i>	AFT29	13	10	
<i>P. fluorescens</i>	AFT36	14	10	
<i>P. fluorescens</i>	948	15	ATCC	
<i>P. fluorescens</i>	31732	16	ATCC	
<i>P. fluorescens</i>	LS107d2	17	14	
<i>P. fragi</i>	71	5	8	milk spoilage
<i>P. putida</i>	345	7	8	milk spoilage
<i>Pseudomonas</i> sp.	113	6	8	milk spoilage
<i>P. xanthophilia</i>	RO28	10	9	raw milk isolate

nonfat dry milk containing 20% glycerol. An inoculum was prepared from a 24-h culture ( $OD_{620} = 0.2$ ) by harvesting the cells with centrifugation ( $6000 \times g$  for 10 min at 4°C), washed three times with sterile saline, and resuspended in sterile saline to an  $OD_{620} = 0.2$ . This was used to inoculate each sterile medium (1% v/v).

The media used in this study were nutrient broth (Difco Laboratories, Detroit, MI), McKellar's minimal medium with lipase inducer (22), UHT skim milk (Gossner Foods, Logan, UT), and Chrisope's medium (5). Lipase inducer for McKellar's medium was prepared from UHT skim milk by acidification with 1 N HCl to pH 4.6 to precipitate the caseins followed by



centrifugation ( $7000 \times g$  for 10 min at  $4^{\circ}\text{C}$ ), and filtration through a Whatman #1 filter paper (Whatman International Ltd., Maidstone, UK). The filtered whey was neutralized to pH 7.0 using 1N HCl and filter-sterilized through a  $0.22\text{-}\mu\text{m}$  syringe filter (Gelman Sciences Inc., Ann Arbor, MI), and stored at  $-70^{\circ}\text{C}$  until required. This was added (1% v/v) to sterile McKellar's minimal medium. Composition of the media is listed in Table 33 (Appendix). Inoculated media were incubated at  $10 \pm 2^{\circ}\text{C}$  with shaking at 200 rpm in a 500 ml flask.

### Sampling

Samples (10 ml) were drawn from each medium at 24 and 50 h and divided into two subsamples. One half was heat treated at  $62.5 \pm 0.5^{\circ}\text{C}$  for 30 min in a water bath and the other half was not given a heat treatment. These two subsamples were used to test protease activity and subsequently referred to as heated and not-heated, respectively. The measurements were done in duplicate.

### Total Plate Count

Total plate counts were determined at both 24 and 50 h before heat treatment of the culture using the standard spread plate method (27).

### Protease Assay

Proteolytic activity was measured by the *o*-phthaldialdehyde (OPA) method of Church et al. (6). This method labels the amino terminal group with OPA released by the protease. This OPA adduct absorbs at 340 nm. A negative control consisting of sterile medium was subtracted from the sample value. Enzyme activity was expressed as activity units/cell (U) (Eq. 1).

$$\text{Activity (U)} = (\text{Average activity (U)} / \text{Average plate count}) \times 10^8 \quad [\text{Eq. 1}]$$

All assays were performed in duplicate and reported as mean values.

Thermostable protease activity was measured as residual activity following heat treatment.

### Protein Quantitation

Protein concentration was determined using a BCA protein assay kit (Pierce Chemical Company, Rockford, IL). A standard curve was constructed using BSA.

### Statistical Analyses

Data were analyzed by analyses of variance using the Minitab statistical software (Release 9.1, Minitab, Inc., State College, PA) in a  $17 \times 4 \times 2 \times 2$  (Eq. 2) factorial with strain, medium, incubation time, and heat treatment as the main effects. The three-way and four-way interaction mean squares were pooled to obtain the error term (Eq. 3) since no replication was done (Pers. comm., Dr. D. Sisson, July, 1998). Least square difference (23) tests were used to calculate the differences between means ( $\alpha < 0.05$ ).

$$Y_{ijklm} = \mu + S_i + M_j + H_l + Sm_{ij} + T_k + St_{ik} + Mt_{jk} + Th_{kl} + SH_{il} + MH_{jl} \quad [\text{Eq. 2}]$$

$$\text{Error} = SMT_{ijk} + SMH_{ijl} + STH_{ikl} + MTH_{jkl} + SMTH_{ijkl} + d_{ijklm} \quad [\text{Eq. 3}]$$

S = strain; M = Medium; T = incubation time;

H = heat treatment; Y = phospholipase activity, d = duplication

## RESULTS

**Influence of Medium, Strain, Incubation Time, and Heat Treatment**

The main effects of medium, strain, incubation time, and heat treatment significantly influenced ( $P < 0.001$ ) protease activity (Table 10). Nutrient broth produced the maximum total activity (1,024 U/h), and skim milk was not significantly different. Chrisope's medium produced the least amount of protease activity (268.4 U/h), and was not significantly different from McKellar's medium (Figure 4A).

TABLE 10. ANOVA for protease production.

Treatment	df	P-value
Medium (M)	3	<0.001
Strain (S)	16	<0.001
Incubation time (I)	1	<0.001
Treatment (T)	1	<0.001
M x S	48	<0.001
M x I	3	<0.001
M x T	3	0.074
S x I	16	<0.001
S x T	16	0.001
I x T	1	<0.001
Error <sup>a</sup>	163	

<sup>a</sup> Error term was the sum of duplication, three-way, and four-way interactions.

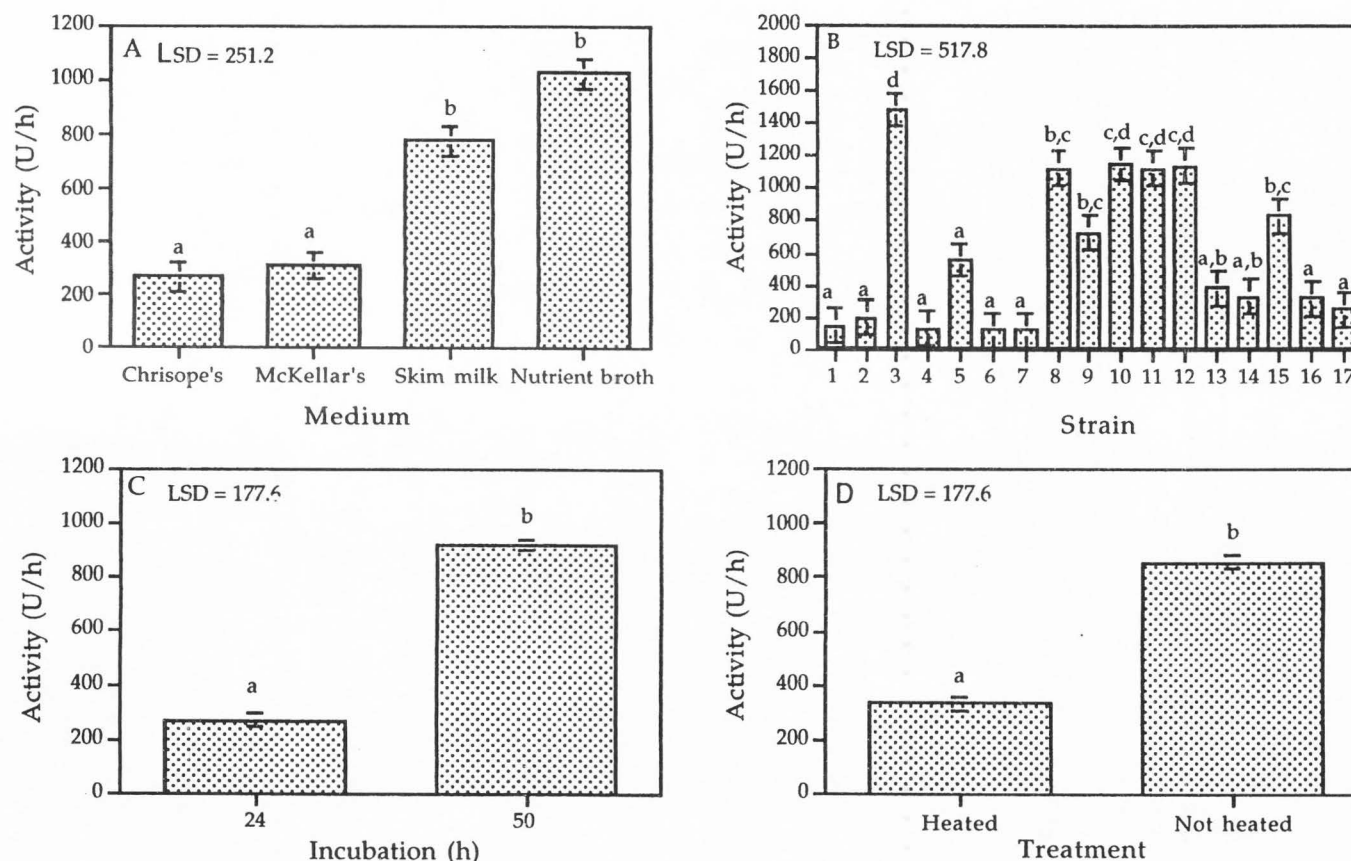


Figure 4. Influence of growth conditions on protease activity. Panel A shows the influence of growth medium, panel B shows the influence of strain (Strain names are provided in Table 9), panel C shows the influence of incubation time, and panel D shows the influence of heat treatment (62.5°C). Activity has been summed over the other three effects for each main effect. Error bars represent standard error of the mean. In some figures the error bar may not show due to the small magnitude. The alphabets represent least significant difference between the means. The same letter on a bar indicates no significant difference.

All of the strains tested produced protease activity, but varied significantly ( $P < 0.001$ ) between strains (Table 10). *P. fluorescens* CHA0 (code 3) produced the most protease activity (1,485 U/h) while *Pseudomonas* sp. 113 (code 6) produced the least at 113 U/h (Figure 4B). Activity significantly ( $P < 0.001$ ) increased with incubation to 50 h (Table 10) (Figure 4C). Protease activity was detected at 24 h (272 U/h), and it decreased by 338% at 50 h (920 U/h) (Figure 4C). Heat-treatment significantly decreased protease activity ( $P < 0.001$ , Table 10). The enzyme lost about 50% activity upon LTLT pasteurization (Figure 4D).

### Interactions and Protease Activity

Growth medium significantly influenced protease activity in all the strains ( $P < 0.001$ , Table 10). Maximum activity was observed in *P. fluorescens* CHA0 (code 3) grown in nutrient broth (3324.8 U/h), whereas another strain of *P. fluorescens* 31732 (code 16) produced only 85 U/h in McKellar's medium. Higher activity was produced in either skim milk or nutrient broth in all of the strains except *P. fluorescens* RO28 (code 10) (Figure 5A). Incubation time significantly increased protease activity in all strains ( $P < 0.001$ ) (Figure 5B). However, comparison of activity of each strain at 24 and 50 h of incubation by the least significance difference method revealed that six strains, *P. fluorescens* CHA0, *P. fragi* 71, *P. fluorescens* 3, RO98, B52, and *P. xanthophilia* RO28 (codes 3, 5, 8 to 11), produced significantly more activity than the others.

Heat stability of the protease varied significantly between strains ( $P = 0.001$ ). A reduction in activity was observed following the heat treatment but all the strains retained some activity (Figure 5C). Residual protease activity following LTLT pasteurization ranged from 5.49% (*Pseudomonas*

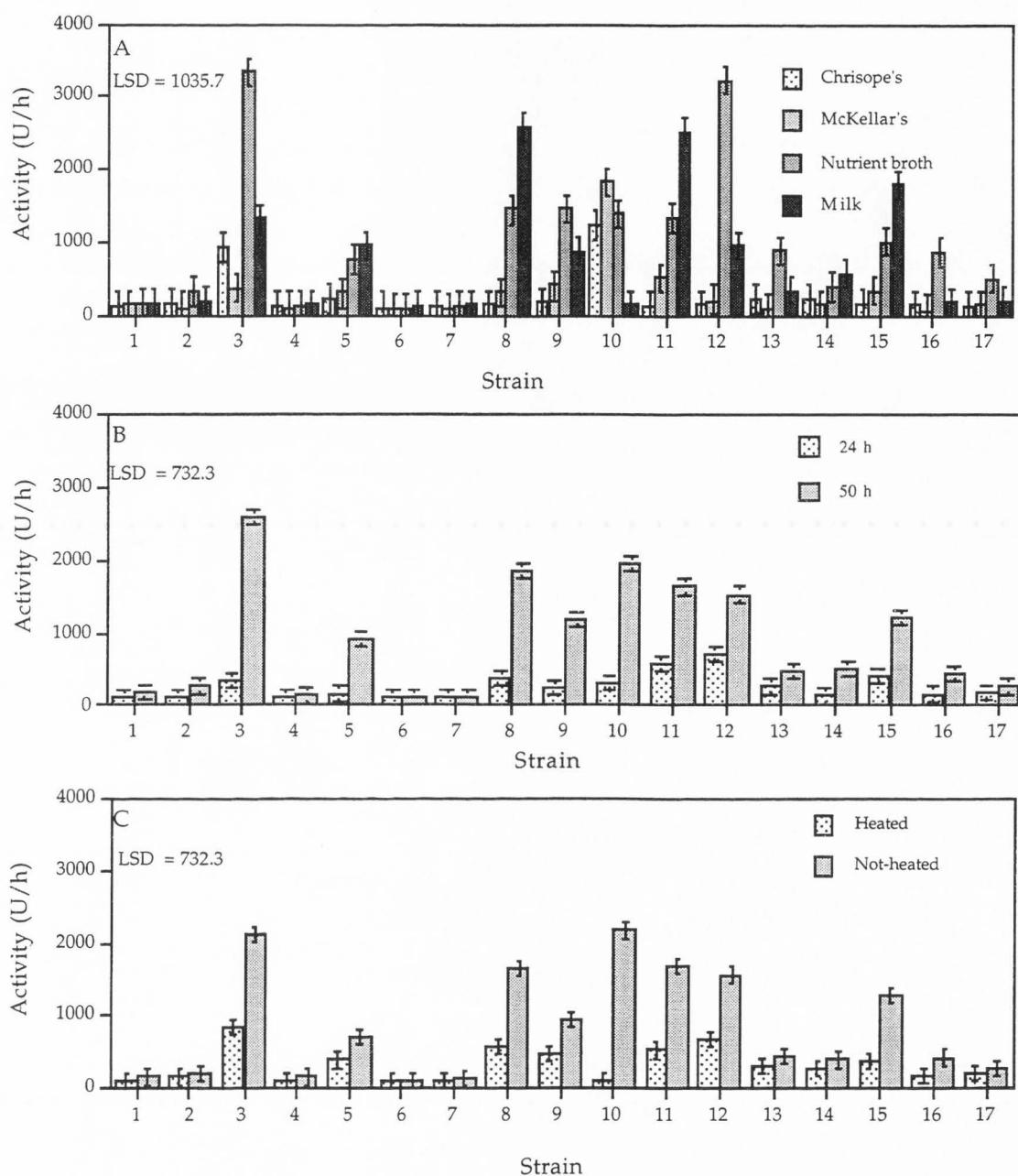


Figure 5. Influence of growth conditions on protease activity. Panel A shows the influence of strain  $\times$  medium, panel B shows the influence of strain  $\times$  incubation time, and panel C shows the influence of strain  $\times$  heat treatment. Strain numbers correspond to the strain names in Table 9. Error bars represent standard error of mean.



*xanthophilia* RO28; code 10) to 91.34% (*P. spp.* 113; code 6). Heat treatment significantly reduced activity in *P. fluorescens* CHA0, 3, B52, RO13, 948, and *P. xanthophilia* RO28 (codes 3, 8, 10 to 12, 15).

Protease activity significantly increased ( $P < 0.001$ , Table 10) with incubation to 50 h in all the media studied (Figure 6A). Activity increased in each medium during the incubation time. However, incubation in skim milk and nutrient broth resulted in a significant increase in activity after 50 h (Figure 6A). Pasteurization did not significantly decrease protease activity during the first 24 h of incubation, but after 50 h of incubation significantly more heat labile activity was produced (Figure 6B).

## DISCUSSION

This investigation was conducted to determine the influence of medium and strain variation for production of protease activity in pseudomonads related to raw milk and dairy products. A factorial experiment with various growth media, strain combinations, and incubation times was done. Additionally, heat stability was determined in the cell-free supernatant. The data were analyzed with an ANOVA using the sum of duplication, three-way and four-way interactions as the error term. This is a conservative approach to the analysis (pers. comm., Dr. D. Sisson, July, 1998) when replication was not conducted. With this in mind, the conclusions drawn based on this analysis will underestimate the influence of each factor, compared to using an error term based on replication rather than duplication.

All the strains used in this study produced protease that was significantly influenced by medium, with nutrient broth and skim milk

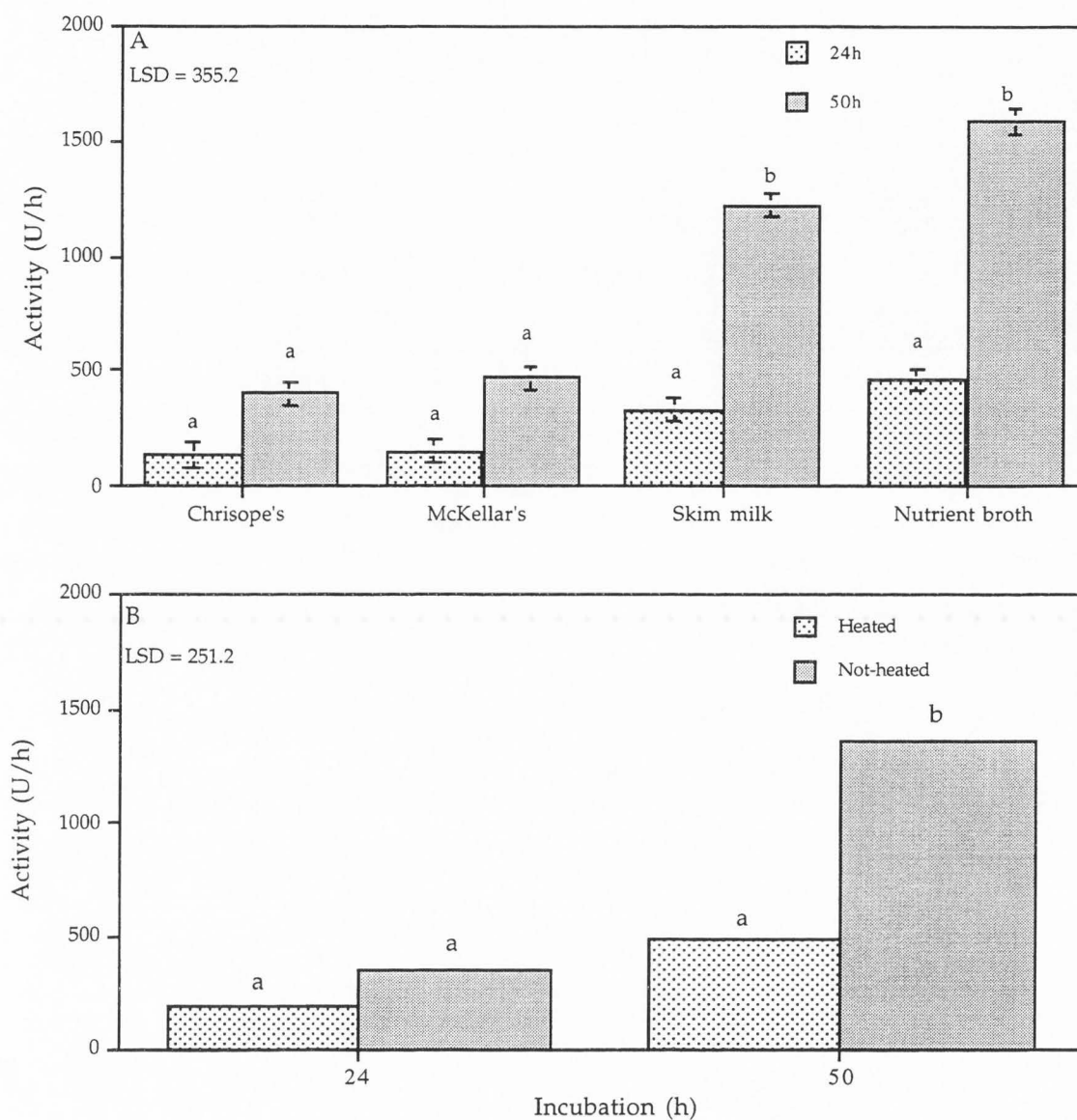


Figure 6. Influence of growth conditions on protease activity. Panel A shows the influence of medium  $\times$  incubation time, and panel B shows the influence of incubation time  $\times$  heat treatment. Error bars represent standard error of mean. Error bars in panel B are too small to be seen. Letters represent least significant difference of the means.

having the highest production (Figure 4A). Nutrient broth is a common laboratory medium for culturing pseudomonads. Nutrient composition of this medium was therefore considered optimal for the growth of the isolates in this study. Chrisope's medium (5) was formulated to yield high phospholipase production in dairy pseudomonads. This was a poor medium for protease production. McKellar's medium (22) induces lipase production in pseudomonads and has a simple composition, which makes it an attractive medium if enzyme purification is needed. Again, this medium produced significantly less protease activity than did skim milk or nutrient broth, despite containing whey proteins.

Significant differences were observed among strains grown in different media. Higher levels of protease activity were observed in either skim milk or nutrient broth for all the strains (Figure 5A). When the variations in media composition are taken into account, it is evident that complex organic compounds enhance proteolytic activity. For example, iron starvation induces protease production (17). This assessment is reasonable for skim milk since it is low in iron or chelated by citrate or the milk proteins (34). The other three media contain no added iron, yet produce little protease activity. Protease production in psychrotrophic milk spoilage bacteria is stimulated by various amino acids and whey proteins and some unidentified small molecular weight compounds from milk (22, 26, 28). In this case, carbon source and free amino acid content may explain the lower protease activity. However, it is difficult to assess the role of these complex media without knowing the exact composition (19). Further research is required to identify these factors in milk so that the problem of heat-stable proteases can be managed more effectively.

Protease activity increased with incubation to 50 h in all the strains tested (Figure 5B). Presumably, the increased activity at 50 h may be a cumulative effect of enzyme activity prior to 50 h of incubation. Alternatively, a second enzyme may be produced in the stationary phase of growth, which begins after about 35 h of incubation (4). In support of this hypothesis, pseudomonad proteases are secondary metabolites produced during late log or stationary phase of growth (15, 22, 34).

All the strains retained a portion of protease activity after pasteurization (Figure 5C). After 24 h of incubation, no significant difference was observed between heated and not-heated samples (Figure 6B). However, after 50 h of incubation significantly more heat labile activity was produced. These observations may indicate that an additional protease is produced in stationary phase and that the enzyme is heat labile. An alternative explanation is that a medium component that stabilizes the enzyme is present at 24 h and is metabolized by 50 h. Protein-protein interactions between the enzyme molecules may also serve this function (13); however, this has not been studied in pseudomonads. In contrast, some psychrotrophic proteases are more thermostable than thermolysin, an extremely thermostable bacterial protease (3, 16). Determining the absolute thermostability of the enzyme was beyond the scope of this work; the intent in this study was to determine the influence of pasteurization on activity at various incubation times.

The strains in this study exhibited differences in their pasteurization stability when grown under similar conditions. For example, when all the isolates were grown in skim milk, residual activity was strain dependent (Figure 5C). Industrially, differences in the heat-stability of the proteolytic

activity in milk will depend on the contaminating strains.

The heat stability of the protease was also dependent on the growth medium. Maximum heat-stable activity was observed in skim milk (Figure 31, Appendix). When *P. fluorescens* 22F was grown in skim milk, tryptone-lactose medium, and milk ultrafiltrate with sodium caseinate, protease produced in milk was more heat stable compared with that in the other two media (30). However, in this study medium did not significantly influence heat stability.

It was determined that protease production significantly increased in skim milk compared to the other media tested, indicating medium composition plays a role in protease production. With this in mind, the impact to milk processing is also variable since milk composition varies during the season (24). Considering that 70 to 80% of raw milk contains psychrotrophic bacteria (1), it is reasonable to suspect that the absolute amount of proteolytic activity and the amount of residual activity after pasteurization will vary during season as well. Increased storage before processing will compound the problem before processing, but it is unclear what role this will have on processed milk because the activity after 50 h was thermolabile.

## CONCLUSIONS

Protease activity was significantly influenced by growth medium and incubation time. Skim milk and nutrient broth produced the highest amount of proteolytic activity. Increasing incubation time also resulted in increased protease activity. However, after 50 h of incubation, the enzyme was heat labile, in contrast to the enzyme produced at 24 h. The observed

differences suggested that different proteases may be produced in stationary phase. Further purification and characterization of the proteolytic enzymes from these strains is required to prove that different enzymes are produced. These findings emphasize the need for limited raw milk storage time, early protease detection, and adequate sanitation to control pseudomonad contamination.

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CHAPTER V  
ISOLATION AND CHARACTERIZATION OF A PROTEASE FROM  
*PSEUDOMONAS FLUORESCENS* RO98

ABSTRACT

*Pseudomonas fluorescens* RO98, a raw milk isolate, was inoculated into McKellar's minimal salts medium and incubated at 25°C for 48 h to allow production of protease. A zinc-metalloacid protease was purified from the cell-free concentrate by anion exchange and gel filtration chromatography. The purified protease was active between 15° and 55°C, and pH 4.5 and 9.0, and was also stable to pasteurization. The enzyme had a pH and temperature optima for activity of 5.0 and 35°C, respectively. It was heat stable with a  $D_{55}$  of 41 min and a  $D_{62.5}$  of 18 h. Molecular weight of the enzyme was estimated to be 52 kDa by SDS PAGE and size exclusion chromatography. Values for  $k_M$  of 144.28, 18.73, 110.20, and 35.23  $\mu M$  were obtained for whole,  $\alpha$ -,  $\beta$ -, and  $\kappa$ - casein with a  $V_{max}$  of 8.26, 0.09, 0.42, and 0.70  $\mu M$ /mg per min, respectively. The enzyme hydrolyzed  $\kappa$ -casein preferentially when incubated with artificial casein micelles.

INTRODUCTION

The widespread use of refrigeration on farms and in milk processing plants improves milk quality by inhibiting the growth of many mesophilic and coliform bacteria, and selects for the growth of psychrotrophic bacteria (11). These bacteria are of concern to the dairy industry because they grow at refrigeration temperatures and produce heat-stable enzymes such as lipase and protease (16, 34). Proteases assume greater significance due to their

remarkable heat stability and contribution to milk spoilage (12, 40). Protease is associated with off odors, flavors, reduced cheese yields, and gelation of UHT milk (6, 36). Consequently, consumer acceptance and shelf-life of milk are reduced (13).

The most common species of psychrotrophic bacteria found in milk is *Pseudomonas fluorescens* (6). Proteases produced by this organism are resistant to both UHT and pasteurization treatments (30, 38). Thermostable proteases are produced by many species of *Pseudomonas* isolated from raw milk (Chapter IV).

Leinmüller and Christopherson (26) partially purified 5 proteases from a single strain of *Pseudomonas*, and Stepaniak and Fox (48) purified three proteases from *Pseudomonas* isolate AFT36. Most of the biochemical properties of these three enzymes were similar including molecular weight. Another study reported the production of four proteases in *P. fluorescens* AR11 but later studies on the same strain (renamed as NCDO 2085) revealed the production of a single protease with characteristics different from previous reports on other *Pseudomonas* strains (3, 12). The observed differences in the properties suggest that multiple proteases are produced in *Pseudomonas* species.

Production of multiple enzymes makes detection and control of proteases in milk difficult. This study was undertaken to determine if multiple proteases were produced within a single strain of *Pseudomonas*. To achieve this objective, protease was isolated from *P. fluorescens* RO98 and characterized.

## MATERIALS AND METHODS

### Strains and Media

Working culture *Pseudomonas fluorescens* RO98 (11), a raw milk isolate, was prepared from frozen culture stocks stored at  $-70^{\circ}\text{C}$  in reconstituted NFDM containing 20% glycerol. Cells were thawed, inoculated into nutrient broth (Difco Laboratories, Detroit, MI), and incubated for 24 h at  $30^{\circ}\text{C}$  with aeration (shaking at 200 rpm). An inoculum for enzyme production was prepared by harvesting the cells by centrifugation ( $6000 \times g$  for 10 min at  $4^{\circ}\text{C}$ ). The cells were washed three times with sterile saline (0.85%). Cells were re-suspended to an optical density of 0.2 at 620 nm in sterile saline. This was inoculated (1%) into a vessel containing 14 L of sterile McKellar's minimal salts medium containing 1% added lipase inducer (28).

Lipase inducer for McKellar's medium was prepared from UHT skim milk by acidification with 1 N HCl to pH 4.6 to precipitate the caseins followed by centrifugation ( $7000 \times g$  for 10 min at  $4^{\circ}\text{C}$ ), and filtration through a Whatman #1 filter paper (Whatman International Ltd., Maidstone, UK). The filtered whey was neutralized to pH 7.0 using 1 N HCl, filter-sterilized through a  $0.22\text{-}\mu\text{m}$  syringe filter (Gelman Sciences Inc., Ann Arbor, MI), and stored at  $-70^{\circ}\text{C}$  until required. This was added (1% v/v) to sterile McKellar's minimal medium. The vessel was incubated at  $25^{\circ}\text{C}$  for 48 h with aeration by constantly purging air through a  $0.2\text{-}\mu\text{m}$  filter (Amicon Inc., Beverly, MA).

### Enzyme Purification

Cells were removed from the culture medium by centrifugation ( $6000 \times g$  for 10 min at  $4^{\circ}\text{C}$ ), and the supernatant was collected. The cell-free supernatant was concentrated to 500 ml by ultrafiltration through a 10-kDa

membrane in a tangential flow ultrafiltration system (Prep/scale TFF ultrafiltration system; Millipore Corporation, Bedford, MA). This was further concentrated in a stirred cell ultrafiltration unit with a 10-kDa membrane (YM10 membrane; Amicon, Inc.) to further reduce the volume to 10 ml. The resulting retentate was filter-sterilized through a 0.22- $\mu$ m syringe filter (Amicon Inc.) and stored at  $-70^{\circ}\text{C}$  until further use. The supernatant concentrate was applied to an anion exchange column (Mono Q column HR 5/5; Pharmacia Biotechnology, Uppsala, Sweden) and eluted with a linear salt gradient of 1 M NaCl in 20 mM sodium phosphate buffer (pH 6.2) with a linear flow rate of 76.5 cm/h.

Fractions were collected at 1-min intervals and individually desalted by washing five times using 20 mM sodium phosphate (pH 6.2) in Centricon-10 ultrafiltration units (Amicon Inc.). Fractions with proteolytic activity were pooled and further purified by size exclusion chromatography on a Superose-12 column (Pharmacia Biotechnology) equilibrated with 20 mM sodium phosphate (pH 6.2) and eluted using the same buffer at a linear flow rate of 38.2 cm/h. Peaks were collected and assayed for protease activity. Purity of the peaks containing protease activity was checked by SDS-PAGE. Contaminating bands were removed by proteolytic degradation by the protease, which was achieved by incubating the preparation at  $4^{\circ}\text{C}$  for a week. Products of proteolysis were removed by washing five times with elution buffer using a 10-kDa centrifugal filter.

### **Protease Assay**

Protease activity was assayed using a highly quenched Bodipy®-FL labeled casein substrate (Enzchek kit; Molecular Probes, Eugene, OR)



according to the manufacturer's instructions. Increase in fluorescence, due to release of fluorescent peptides by proteolysis, was followed in a spectrofluorophotometer (RF 1501; Shimadzu, Columbia, MD) at an excitation wavelength of 480 nm and emission wavelength of 520 nm. The increase in fluorescence obtained due to hydrolysis of the protein was expressed as relative fluorescence units (RFU/h).

### **Protein Quantitation**

Protein concentration was monitored continuously during chromatography by measuring absorbance at 280 nm (System Gold, Beckman Instruments, CA). For other experiments involving protein concentration measurements, a BCA protein assay kit was used according to the manufacturer's instructions (Pierce Chemical Company, Rockford, IL). Bovine serum albumin was used to construct a standard curve.

### **Molecular Weight Determination**

Molecular weight of the purified protease was estimated by SDS-PAGE and size-exclusion chromatography. Electrophoresis was conducted on a Phast system (Pharmacia Biotechnology) in reducing conditions using an 8 to 25% gradient polyacrylamide phast gel (Pharmacia Biotechnology). Samples were prepared in 50 mM Tris-HCl buffer (pH 6.8) containing 2.5% SDS and 1%  $\beta$ -mercaptoethanol. A 10-kDa protein ladder was used to estimate molecular weight (Life Technologies, Gaithersburg, MD). Protein bands were visualized by silver staining (Plusone; Pharmacia Biotechnology).

Native molecular weight of the enzyme was determined by size-exclusion chromatography. The enzyme was loaded on a Superose-12 size-exclusion column (HR 10/30; Pharmacia Biotechnology) and eluted with 20

mM sodium phosphate (pH 6.5) at a linear flow rate of 38.2 cm/h. The column was calibrated with ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa),  $\beta$ -lactoglobulin (36 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa) (Pharmacia Biotechnology).

### **Influence of Temperature and pH**

The influence of temperature on protease activity was determined by assaying activity at temperatures ranging from 5 to 70°C in 20 mM Tris-HCl buffer (pH 7.4). Buffers were made and preincubated at the respective temperature prior to the assay.

The optimum pH for the activity of the enzyme was determined by assaying activity in 50 mM MES (pH 4.0 to 7.3), 50 mM Tris-HCl (7.2 to 8.9), or 50 mM carbonate (8.5 to 10.6). All samples were incubated for 1 h at 30°C.

### **D-value Determination**

Heat-stability of the enzyme was determined at 55 and 62.5°C. The enzyme was diluted to a concentration of 12.8  $\mu$ g/ml in Jenness Koops buffer (21). Two hundred microliters of this was dispensed into thin-walled PCR tubes and heat treated in a thermocycler (MJ Research Inc., Watertown, MA). Duration of treatment was different for the two temperatures. The enzyme was heated for 0.25 to 80 min at 55°C and 24 h at 62.5°C. Residual activity was expressed as a percentage of the untreated sample. A negative control containing only substrate was maintained for each time point.

### Inhibition Studies

Inhibitors were used to determine the class of protease (9). The inhibitors tested were EDTA (Ethylene diamine tetraacetic acid) (divalent cation chelator), EGTA (ethylene glycol-bis- $\beta$ -aminoethyl ether N, N, N', N'-tetraacetic acid) (specific chelator of  $Mg^{2+}$  ions), *o*-phenanthroline (specific chelator of  $Zn^{2+}$  ions), phosphoramidon (N-( $\alpha$ -rhamnopyranosyloxy-hydroxyphosphinyl) Leu-Trp) (serine protease inhibitor), PMSF (Phenyl-methyl-sulfonyl-fluoride) (serine protease inhibitor), Pepstatin A (Isovaleryl-val-val-sta-ala-sta; sta=statine) (acid protease inhibitor), and leupeptin (Acetyl-leu-leu-arg-ala) (thiol protease inhibitor) (Sigma Chemical Co., St. Louis, MO). The inhibitors were added at various levels to a reaction mixture containing 5  $\mu$ l (approximately 1.28  $\mu$ g) of the pure enzyme, and incubated for 4 h at 30°C. Negative controls containing all components of the reaction mixture except the enzyme was used for each inhibitor at all the levels tested. This was used in activity adjustments. The corrected activity was expressed as a percentage of the positive control tube that did not contain any inhibitors. Dunnett's test was used to determine significant differences of the treatments from control (Minitab Statistical Software, Release 9.1, Minitab, Inc., State College, PA ).

### Determination of Kinetics of Enzyme Catalysis

The enzyme was incubated with various concentrations of four different fluorescently-labeled substrates. Bodipy®-FL-labeled whole casein was used to determine the  $k_M$  of the enzyme against whole casein (Enzchek, Molecular Probes).  $\alpha$ -,  $\beta$ -, and  $\kappa$ - caseins (Research Organics, Cleveland, OH), were labeled with tetramethyl rhodamine, Texas red, and fluorescein,

respectively (custom synthesis; Molecular Probes). The reaction mixture consisted of 0.7 to 175  $\mu\text{M}$  of each casein solution in 20 mM Tris-HCl (pH 7.8). The reaction was started with the addition of 5  $\mu\text{l}$  (approximately 1.28  $\mu\text{g}$ ) of enzyme solution and incubated at 30°C. Proteolytic degradation was followed by measuring fluorescence in a RF1501 spectrofluorophotometer (Shimadzu) at the following excitation and emission wavelengths; 540/565; 580/605; and 480/520 for  $\alpha$ -,  $\beta$ -, and  $\kappa$ - caseins, respectively. Slopes were calculated from the initial 3 min of reaction and Michaelis-Menton constants (46) were determined using nonlinear regression (Statistica Software, StatSoft, Tulsa, OK).

#### **Preparation and Hydrolysis of Fluorescent Artificial Casein Micelles**

Artificial casein micelles were prepared by the method of Schimdt et al. (44) using the labeled  $\alpha$ -,  $\beta$ -, and  $\kappa$ -caseins with some modifications. The fluorescently-labeled caseins were dissolved in 0.07 M NaCl (pH 8.6) after which the pH was adjusted to 7.2 using 0.1 M HCl. The casein micelle solution was diluted 1:100 in 50 mM Tris-HCl buffer (pH 7.0). Activity was monitored continuously over a 10-min incubation period at 30°C in a spectrofluorophotometer, immediately following the addition of 5  $\mu\text{l}$  protease (approximately 1.28  $\mu\text{g}$ ) (Shimadzu). Hydrolysis of each casein fraction in the micelle was determined by measuring fluorescence at their respective excitation and emission wavelengths. Slopes were calculated from the initial 2 min of reaction.

#### **Electron Microscopy**

Formation of micelles was determined by transmission electron

microscopy using the method of McManus and McMahon (33). The micelle solution (50  $\mu$ l) was placed on a parlodion coated copper grid (Electron Microscope Sciences, Fort Washington, PA) and incubated at room temperature for 10 min in order to allow adsorption of the proteins to the grid. The unadsorbed proteins were removed by rinsing three times in distilled water followed by staining in 200  $\mu$ M uranyl oxalate (50). The samples were quick frozen in chilled Freon 113 and transferred to liquid nitrogen and freeze-dried in an IBS-100 lyophilizer (VCR Group, San Francisco, CA). Images were made on a Zeiss 902 CEM camera at 80 kV (LEO, Zeiss One, Thornwood, NY) and recorded on Kodak SO-163 film (Eastman Kodak Co., Rochester, NY). Electron micrographs of skim milk were produced using the same procedure and used for comparison.

### **Electroblotting and Microsequencing**

Electrophoresis (SDS-PAGE) was done under reduced conditions using a 160 x 175 x 0.75 mm discontinuous polyacrylamide gel with 5% stacking (pH 6.8) and 14% resolving (pH 8.8). Samples and buffers were prepared according to the method of Laemmli (24). The gel was run in a Protean II Xi electrophoresis cell (Biorad Laboratories, Hercules, CA) for 3 h at a constant current of 30 mA. Following SDS-PAGE, the proteins were blotted to a polyvinylidene fluoride membrane (Immobilon-PSQ; Millipore Corporation, Bedford, MA) overnight in a Trans-blot cell (Biorad Laboratories, Hercules, CA) at a constant voltage of 14 V. The blot was developed in Coomassie stain (9) and the protease band was excised and the N-terminus was sequenced (Univ. Texas Health Center, Tyler, TX). The sequence obtained was compared with other protein sequences in the Swiss-Prot database.

## RESULTS

### Enzyme Purification

A single peak with protease activity eluted from the anion exchange column prior to the NaCl gradient, indicating that the protein did not adsorb to the resin. Chromatography with an anion exchange column was, however, useful in the removal of other proteins that interacted with the resin and eluted with the salt gradient (Figure 7A). The partially purified preparation was concentrated and applied to a size-exclusion column. The enzyme eluted as a single peak (Figure 7B). The enzyme was further purified using size-exclusion and ultrafiltration. These procedures resulted in an 8-fold increase in specific activity with a 27.8% yield (Table 11).

Contaminating proteins were degraded during incubation at 4°C for one week followed by washing with the elution buffer five times using a 10-kDa centrifugal filter. A single protein (Figure 8) with an approximate molecular mass of 52 kDa was obtained by SDS PAGE and size exclusion chromatography.

### Temperature and pH Optima

The pure enzyme was active over a wide range of temperature from 15 to 60°C with an optimum of 35°C (Figure 9A). An optimum pH for activity of 5.0 was determined (Figure 9B).

### Inhibition Studies

Among the inhibitors studied, EGTA, *o*-phenanthroline, and EDTA, all chelators of divalent cations, inhibited activity (Table 12), indicating that



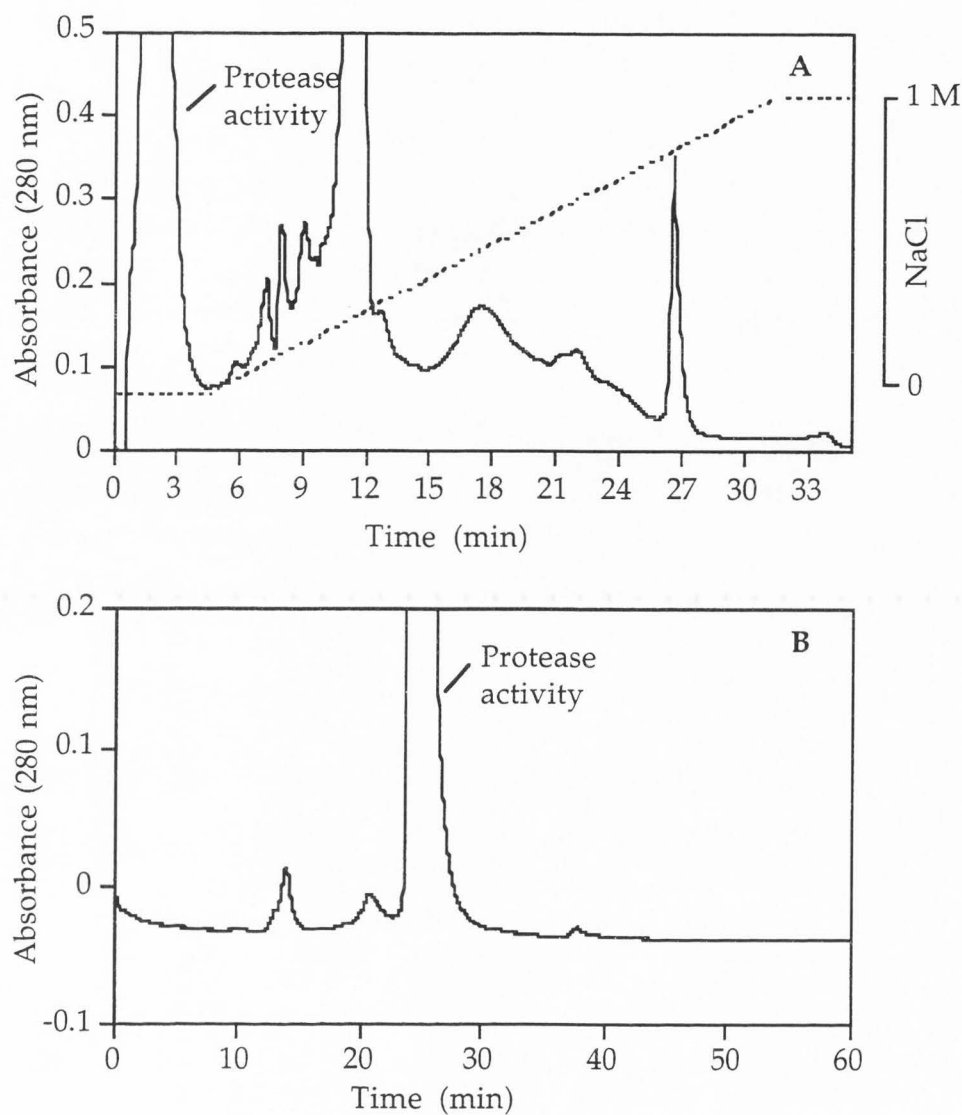


Figure 7. Anion-exchange chromatography (Panel A) and size-exclusion chromatography (Panel B) of concentrated supernatant from *P. fluorescens* RO98.

TABLE 11. Purification of protease from *P. fluorescens* RO98.

Purification step	Total protein (mg)	Total activity (U <sup>a</sup> )	Specific activity (U <sup>a</sup> /mg)	Purification factor	Yield (%)
Crude concentrate	6.6	394.1	59.2	1.0	100
Anion exchange (Mono Q)	3.0	381.6	126.8	2.1	96.8
Size exclusion (Superose-12)	0.5	227.8	493.2	8.3	57.8
Ultrafiltration	0.1	109.7	3656.7	61.8	27.8

<sup>a</sup>One unit is defined as 1 RFU/min.

the enzyme was a metalloprotease. Among the three metal-chelating inhibitors, complete inactivation was observed with *o*-phenanthroline, which is a specific chelator of zinc ions. Loss in activity (40%) was observed in the presence of 0.5 mM pepstatin, an acid protease inhibitor.

Phosphoramidon, PMSF, and leupeptin had no inhibitory effect on protease activity.

### Heat Stability

The estimated D-value for the destruction of this enzyme was 41.3 min at 55°C (Figure 10A). A D-value of 18.1 h was determined at 62.5°C. A 30 min treatment at 62.5°C, which is a typical batch pasteurization treatment, resulted in 52.4% loss of activity.

### Kinetic Parameters

Data collected for high substrate concentrations were not used in the determination of kinetic parameters because a reduction in activity was

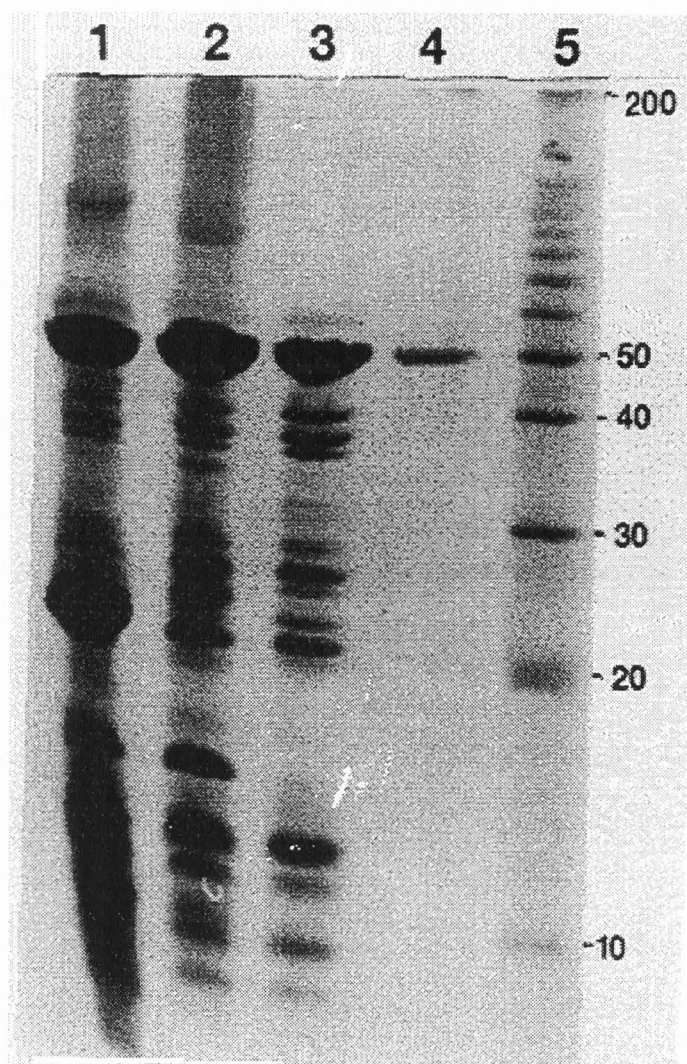


Figure 8. SDS-PAGE analysis of protease purification from *P. fluorescens* RO98. Lanes: 1, Crude concentrate (10 kDa retentate); 2, mono Q; 3, size-exclusion; 4, peptide wash; 5, 10 kDa protein ladder, numbers on the right side indicate the molecular weight in kDa.

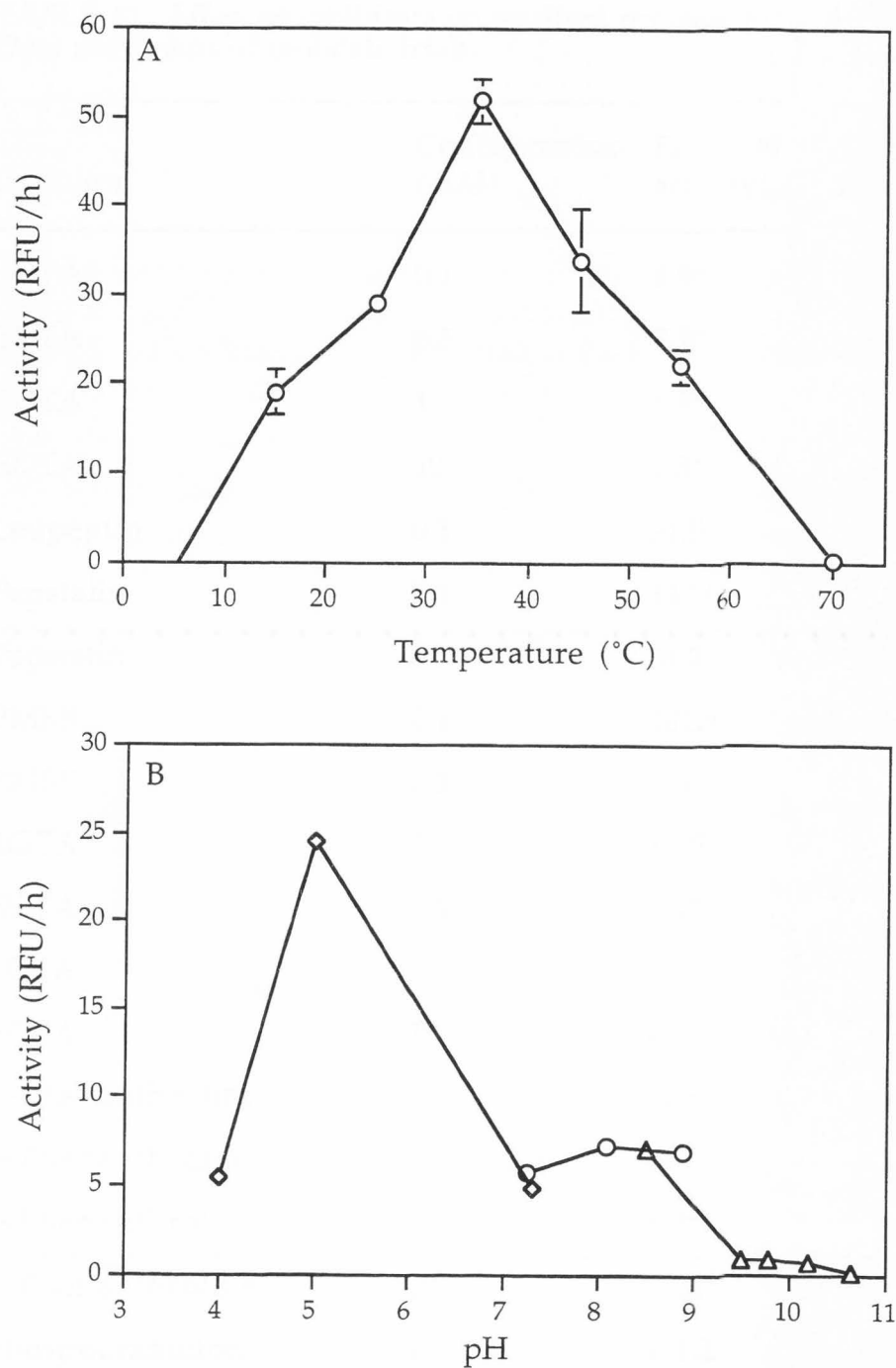


Figure 9. Temperature (Panel A) and pH optimum (Panel B) of protease. Buffers used to determine pH optimum; MES ( $-\diamond-$ ), Tris-HCl ( $-o-$ ), and carbonate ( $-\Delta-$ ). Results are means of two replications. Error bars indicate the standard deviation of the means.

TABLE 12. Effect of inhibitors on purified protease activity.  
Data are means of replicate trials.

Inhibitor	Concentration (mM)	Residual activity(%)
EDTA	0.1	6.9*
EDTA	0.5	7.3*
EDTA	1	6.9*
EDTA	10	1.3*
Leupeptin	0.1	91.5
Pepstatin	0.1	140.6
Pepstatin	0.5	61.2
PMSF	0.1	101.9
PMSF	0.5	109.3
EGTA	0.1	13.5*
EGTA	0.5	9.4*
EGTA	1	7.4*
EGTA	10	4.7*
<i>o</i> -Phenanthroline	0.1	74.5
<i>o</i> -Phenanthroline	0.5	28.0*
<i>o</i> -Phenanthroline	1	6.8*
<i>o</i> -Phenanthroline	10	-0.2*
Phosphoramidon	1	101.2
Control	No inhibitor	100

\*significantly different from control

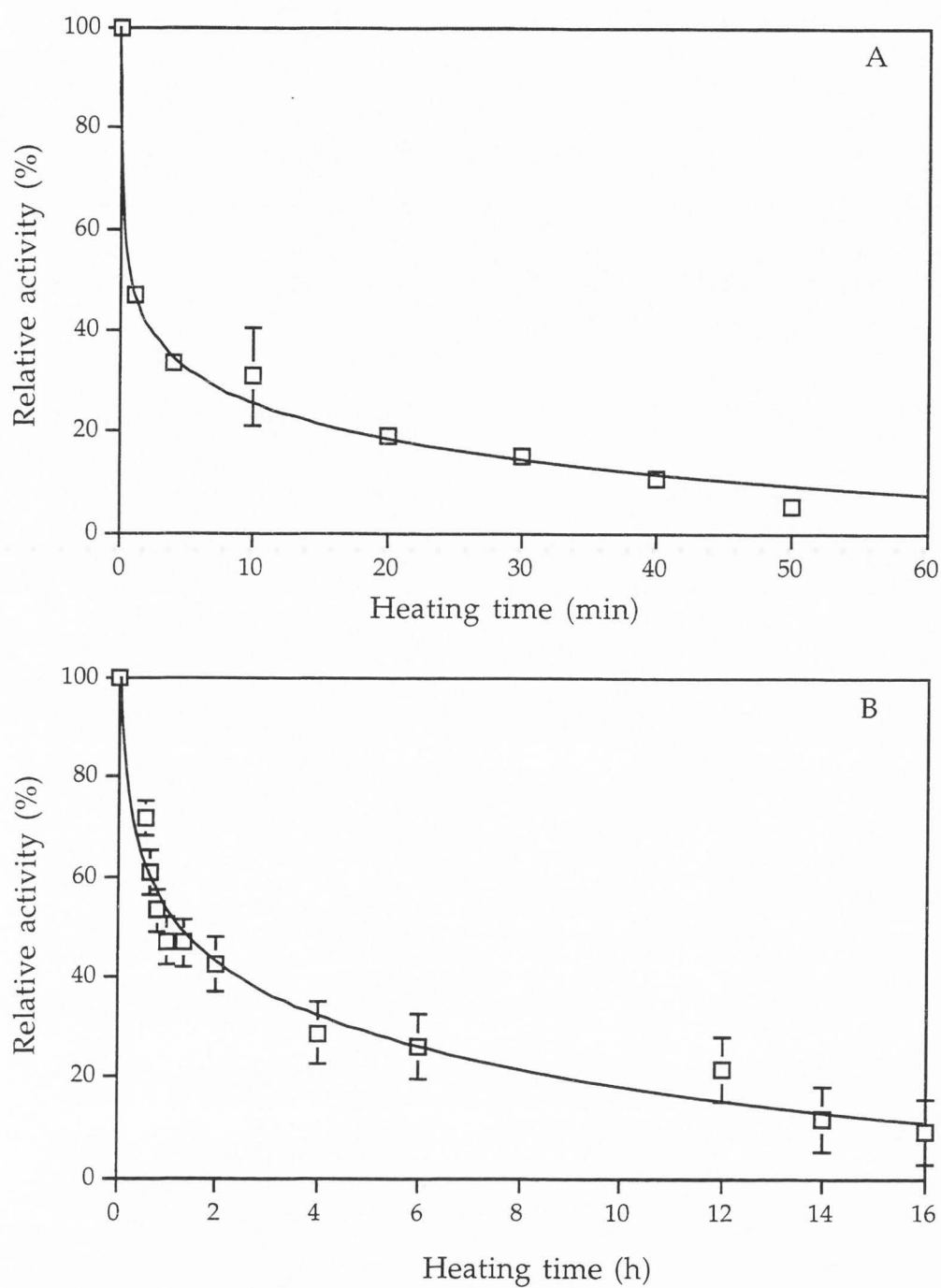


Figure 10. Loss of protease activity at 55°C (Panel A) and 62.8°C (Panel B). Error bars represent standard deviation of the means from two replications.



observed at these concentrations (Figure 11). Values for  $k_M$  were obtained for whole,  $\alpha$ -,  $\beta$ -, and  $\kappa$ - casein, as were  $V_{max}$  estimates (Table 13).

### Casein Micelle Hydrolysis

Electron micrographs of artificial casein micelles were different than native skim milk micelles (Figures 12A, B). The differences may be attributed to the modifications in the protocol and the technique used in sample preparation for electron microscopy.

Kappa-casein was degraded rapidly within the micelle with a slope of 0.14, followed by  $\beta$ -casein with a slope of 0.04. Alpha-casein was hydrolyzed much slowly and yielded a slope of only 0.03 (Figure 13).

### Microsequencing

The N-terminal sequence determined was; Val-Gln-Asp-Glu-Asp-Ala-Phe-Gly-Tyr-Val-Arg-(Xxx)-Ile-Thr-Ser-Arg-Gly-Ala-Phe-Gly. Comparison of the sequence with those in the Swiss-Prot and Genbank databases revealed a 76% similarity to a hypothetical Vaccinia virus protein and 52% similarity to an alkaline protease zymogen produced by *Saccharomyces cerevisiae*.

## DISCUSSION

This study was conducted to investigate the production of multiple proteases in *Pseudomonas* (Chapter V). Differences in the properties of the enzyme with strain, incubation time, medium of growth, and heat treatment indicated that multiple proteases may be produced within a single strain. To verify this hypothesis, protease purification from the concentrated supernatant was done with *P. fluorescens* RO98.

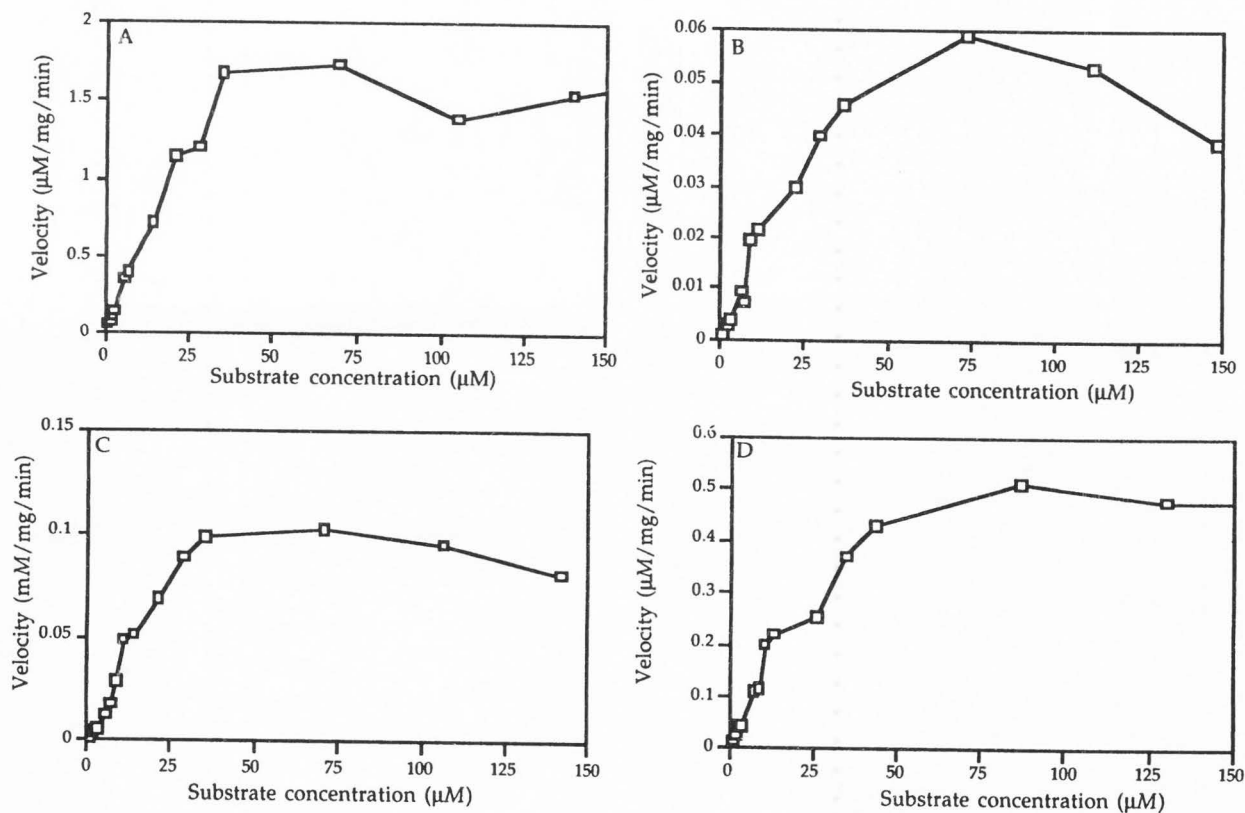


Figure 11. Effect of substrate concentration on the rate of enzyme catalyzed hydrolysis of whole-casein (Panel A);  $\alpha$ -casein (Panel B);  $\beta$ -casein (Panel C);  $\kappa$ -casein (Panel D).

TABLE 13. Kinetic parameters for pure protease.

Casein fraction	$V_{\max}$ ( $\mu\text{M}/\text{mg}/\text{min}$ )	$k_M$ ( $\mu\text{M}$ )
Whole casein	8.26	144.28
$\alpha_{s1}$ -casein	0.09	18.73
$\beta$ -casein	0.42	110.20
$\kappa$ -casein	0.70	35.23

Ion exchange and size exclusion chromatography yielded a single peak with protease activity (Figure 7). This resulted in a partially purified enzyme contaminated with other proteins. Fortunately, after storage at 4°C for a week, the other proteins were degraded by the protease, resulting in a pure protease preparation after ultrafiltration with a 10-kDa membrane. This strategy, while it produced a pure protease, was not optimal for purification. For example, about 80% of the protein was lost as well as about 50% of the activity degraded during storage. The remaining protease may have degraded other proteolytic enzymes present in the mixture. Therefore, production of multiple enzymes cannot be ruled out, but it can be assumed that at least one protease dominates the mixture and that was the one isolated in this study. Presumably, this would be the case with milk that is stored before processing. Hence, it was worthwhile to characterize and study this protease, despite the possibility that other enzymes exist in this strain.

Implications of protease activity on milk quality are many. The isolated protease exhibited some unique properties; it was active over a wide temperature (5 and 55°C) and pH range (pH 4.5 and 9.0). These ranges include many of the storage conditions for fluid and fermented dairy products. For

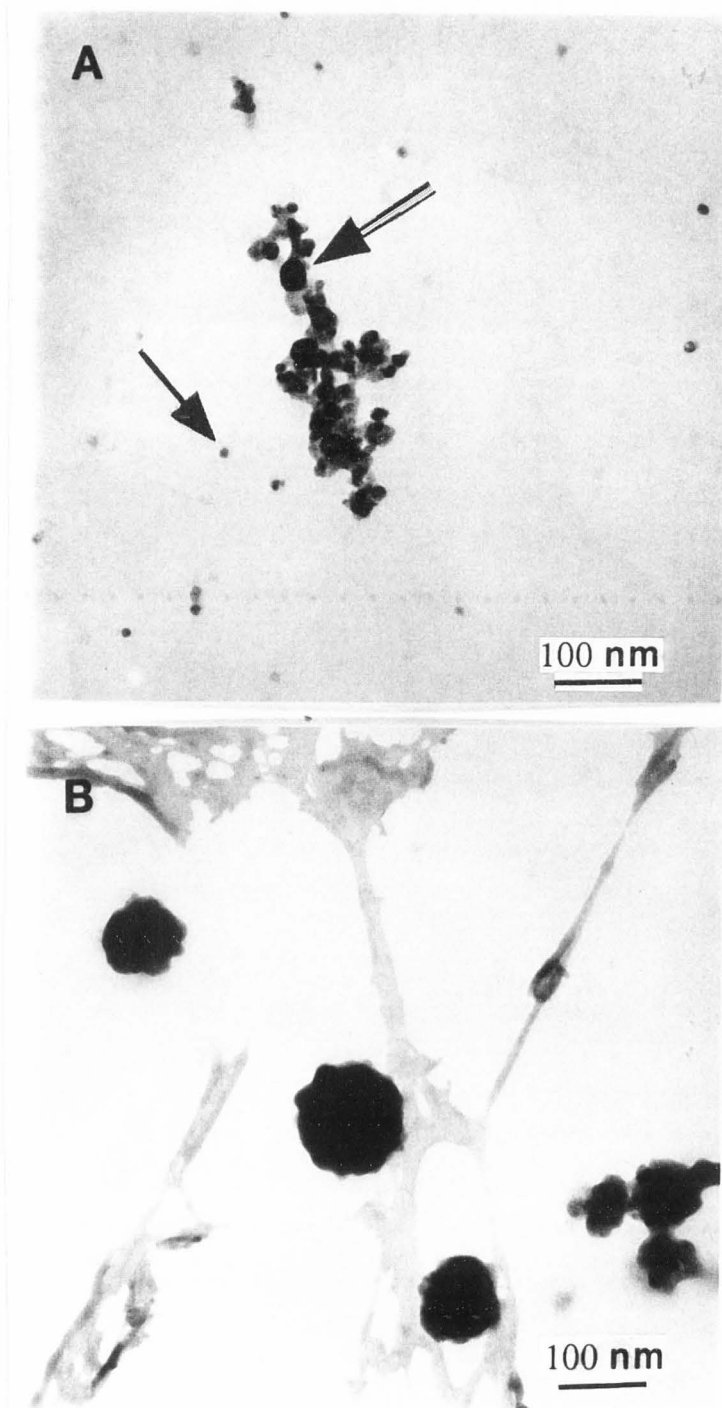


Figure 12. Transmission electron micrograph of casein micelles; labeled artificial casein micelles (Panel A) and native micelles in skim milk (Panel B). Single-line arrow points toward submicelles and double-line arrow points toward larger micelles.

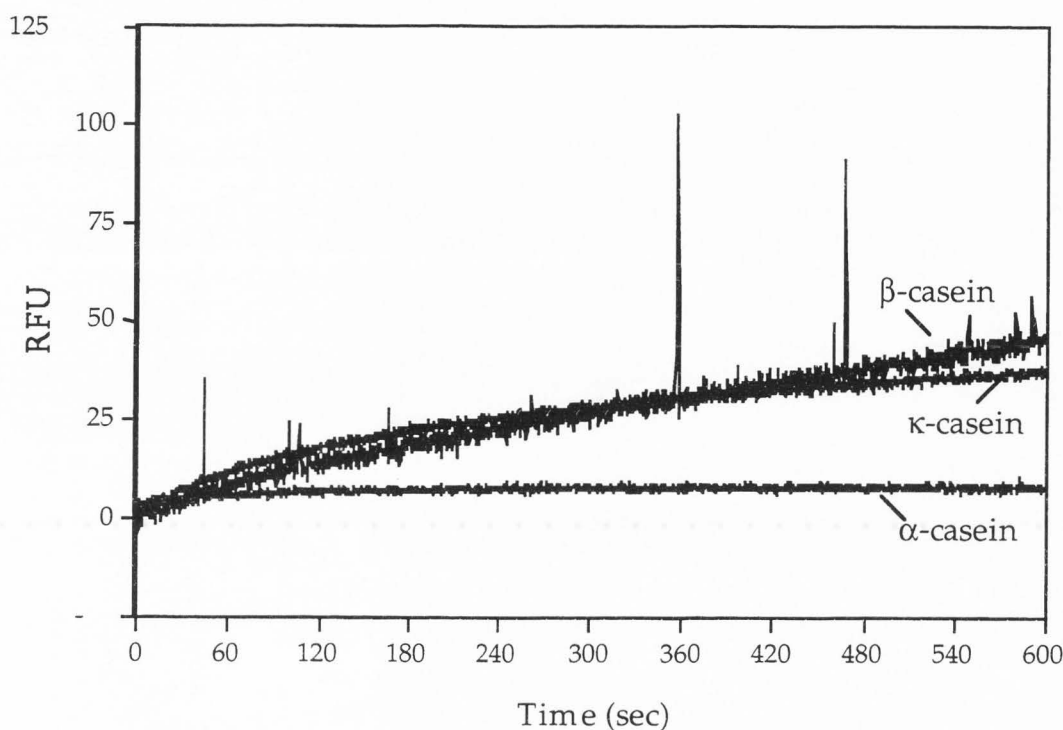


Figure 13. Hydrolysis of fluorescent artificial casein micelles.

example, Cheddar cheese is stored at 5–13°C for many months and has a pH of about 5.2, the optimum pH for this enzyme. These data combined lead one to suspect that this enzyme may be active during cheese ripening.

Considering fluid milk, with a pH of 6.8 and a storage temperature of 4 to 8°C, the enzyme would be less active in milk than in Cheddar cheese.

Inhibition studies showed that the isolated enzyme was a metallo–acid protease. It was also evident that this enzyme required  $\text{Zn}^{2+}$  for its activity because *o*-phenanthroline, a  $\text{Zn}^{2+}$  chelator, completely inhibited protease activity. The enzyme also required  $\text{Ca}^{2+}$  because EGTA, which specifically chelates  $\text{Ca}^{2+}$ , caused an inhibition of activity. Based on these findings, this enzyme can be classified as a zinc–metalloacid protease (13, 15, 22, 43, 45). In

other zinc-metalloproteases,  $\text{Zn}^{2+}$  is in the active site, and  $\text{Ca}^{2+}$  is required for the formation and stabilization of protein structure (29, 45). It is likely that role of  $\text{Zn}^{2+}$  and  $\text{Ca}^{2+}$  is the same for this enzyme. Milk contains divalent cations such as zinc (0.38 mg/deciliter) and calcium (117 mg/deciliter); therefore, the enzyme will be active in milk (49). A factor regulating activity may be the competition for the metal ions with the other proteins in milk. If the milk proteins have a higher binding constant than this protease, the milk proteins will "hold" the metal. Perhaps degradation of the milk proteins is a strategy to compete for the metals in milk.

The pure enzyme was thermostable at both 55 and 62.5°C (Figure 10). A 30 min treatment at 62.5°C in Jenness-Koops buffer (21), which is a typical batch pasteurization treatment, resulted in a 52.4% loss in activity. Stepaniak and Fox (48) report D-values of 6.0 and 5.2 h at 55 and 62.5°C, respectively for *P. fluorescens* AFT36 protease. The calcium present in the buffer may have stabilized the enzyme by the formation of intramolecular bridges rendering the protein structure less flexible and, consequently, less susceptible to proteolysis (14, 20). Ionic calcium has a stabilizing effect against thermal inactivation in several proteases including thermolysin and subtilisin (14, 18, 26, 27). The purified protease was less heat stable when heated at 55°C unlike the protease from *P. fluorescens* AFT36 (48). The decreased heat resistance of proteases to heating at temperatures below pasteurization is termed as low temperature inactivation (LTI) (19). Low temperature inactivation phenomenon may be due to auto proteolysis (10, 17, 23, 38, 45). It could be due either to intermolecular proteolysis of partially unfolded protease molecules (2, 14, 38, 45) or to intramolecular autoproteolysis of a partially unfolded protease molecule (48). Therefore, once the enzyme is produced, it



will survive milk processing and bring about proteolytic spoilage of processed milk during refrigerated storage.

Velocity of whole,  $\alpha$ -,  $\beta$ -, and  $\kappa$ -casein hydrolysis decreased with concentrations of substrate greater than 100 mM (Figure 11). The observed decrease in velocity of the hydrolytic reaction at higher concentrations can be explained by either self-aggregation or micelle formation of the caseins making the bonds less available for hydrolysis. Among the individual casein types, the magnitude of  $k_M$  increased in the following order:  $\alpha$ ->  $\kappa$ ->  $\beta$ -caseins -> whole casein (Table 13). Self-association of  $\alpha_{s1}$ -casein is dependent on pH and ionic strength, ionic strength greater than 0.01M at neutral pH enhances aggregation (42).  $\beta$ - and  $\kappa$ -casein exhibit amphiphilic self-association to form a micelle. Association of  $\beta$ -casein is characterized by a critical micelle concentration ranging from 0.3 to 0.7 mg/ml depending on the temperature and ionic strength. In this study  $\beta$ -casein was used from 0.71 to 148  $\mu$ g, suggesting a micelle was formed during the experiments to define the kinetics. A critical micelle concentration of 0.2 to 0.5 mg/ml is observed for  $\kappa$ -casein, which increases with decreasing ionic strength (42). The concentration used in this study was 0.87 to 174, indicating that micelles would also form with this substrate.

The caseins used in the present study were labeled with fluorescent labels on the lysine residues. The number of lysine residues in the caseins is variable: 14 to 24 in  $\alpha$ -casein, 11 in  $\beta$ -casein, and 9 in  $\kappa$ -casein (49). Furthermore, the labeling will change the charge, conformational state, and availability of the susceptible bonds (which is unknown for this protease). Therefore, the kinetic parameters obtained for the caseins used in the present investigation do not reflect the hydrolysis of unlabeled caseins. However,

data on hydrolysis of each casein fraction within a micelle are more practical because the caseins exist as micelles in milk (49).

To establish the preference of the protease for each casein fraction within a micelle, artificial casein micelles were prepared with the labeled caseins at the ratios found in milk. Electron micrographs of the artificial casein micelles were different from skim milk micelles. Smaller submicellar structures were found, probably with  $\kappa$ -casein on the surface (5, 31, 32, 41). Larger irregular structures were also found and these may have been formed from the sub-micelles. Due to problems in dissolving the labeled caseins for micelle formation as outlined by Schmidt (44), the preparation protocol was modified. Presumably, this problem was due to the fluorescent label, which lead to differences in the micelle formation observed in the micrographs.

Additionally, the artificial micelles were used to determine hydrolysis of the caseins.  $\kappa$ -Casein was hydrolyzed preferentially compared to  $\beta$ - and  $\alpha$ -casein when incubated with the pure protease. This is logical because  $\kappa$ -casein is on the surface of the micelle and its hydrolysis results in the destabilization and subsequent coagulation of milk because  $\kappa$ -casein stabilizes the micelle (5, 31, 32, 41). Temperature of incubation may also determine which one of the three casein fractions will be hydrolyzed.  $\beta$ -casein is expected to be in the micelle at the assay temperature (30°C). It may be possible that  $\beta$ -casein will be hydrolyzed at a greater rate during refrigerated storage of milk because  $\beta$ -casein exits the micelle at lower temperature (49). Disturbance of the casein micelle integrity by proteolysis affects curd formation in the manufacture of various dairy products such as cheese, yogurt, and sour cream, leading to decreased cheese yields, unacceptable texture, flavor, and eventually poor quality products (4, 7, 8, 25, 37).

Proteolysis leads to the formation of bitter off-flavors, and gelation of milk during storage (1, 7, 30, 35, 39, 47).

## CONCLUSIONS

A single, monomeric protease with a molecular weight of approximately 52 kDa was isolated from *P. fluorescens* RO98 with an N-terminal amino acid sequence that was similar to an alkaline protease. The isolated enzyme was a zinc metalloprotease with an optimum pH and temperature for activity of 5.0 and 35°C, respectively. The enzyme had a preference for  $\alpha$ -casein as indicated by its low  $k_M$  when tested with individual caseins. However, it hydrolyzed  $\kappa$ -casein preferentially when incubated with artificial micelles. This property combined with the high thermal stability makes this enzyme an interesting subject for future research on milk spoilage. Detection and control of protease activity are critical because the enzyme is able to resist pasteurization and to, remain active at the pH and temperature of fluid and fermented dairy products. The impact in fermented products is unknown. The impact of this enzyme may be detrimental in fluid milk for long shelf-life dairy products.

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CHAPTER VI  
RAPID DETECTION OF EXTRACELLULAR *PSEUDOMONAS*  
*FLUORESCENS* PROTEASE

ABSTRACT

Psychrotrophic bacteria, mainly *Pseudomonas fluorescens*, dominate raw milk and produce heat-stable extracellular enzymes such as protease during growth in milk. Sensitive proteolytic assays exist for use in buffer systems, but they are not suitable for use in milk due to the competition of milk proteins with the substrate hydrolysis. Existing immunoassays have a sensitivity of 0.24 ng/ml for total protein, but take about 6 h to complete. This work describes a method to detect protease in 2 h. Polyclonal antibodies to a purified *Pseudomonas fluorescens* protease were immobilized on 2.8- $\mu$ m diameter paramagnetic-polystyrene and 7-mm diameter ceramic beads with a poly-threonine spacer between the antibody and the surface of the bead. The enzyme was captured, removed from the sample, and detected by a solid phase ELISA to determine the total enzyme present. Simultaneously, protease activity was tested on the bead surface. Using this assay, both active and inactive forms of protease were detected at 427 ng.

INTRODUCTION

The widespread use of refrigeration on farms and in milk processing plants improves milk quality by inhibiting the growth of many mesophilic and coliform bacteria (5), but selects for the growth of psychrotrophic bacteria. Most of the psychrotrophic bacteria are capable of producing extracellular enzymes that degrade milk constituents and therefore pose a threat to milk

quality. Once extracellular proteases are present in milk, it is nearly impossible to inhibit their activity, and detection of the enzymes assumes greater importance (10).

Diffusion methods using skim milk agar, gelatin agar, and elastin agar are often used in the dairy industry for the detection of protease activity (7, 8). The requirement for extended incubation and lack of sensitivity of these methods restricts their use to qualitative purpose (6). Other methods involve the assay of proteolytic products (4) that are determined by using amine group specific reagents such as *o*-phthaldialdehyde (3), fluorescamine (10), and trinitrobenzenesulfonic acid (19). Alternatively, peptides formed due to hydrolysis can be precipitated by trichloroacetic acid and quantitated by UV absorption (12), or estimated by colorimetric means using Folin-Ciocalteau reagent (7) or bicinchoninic acid (18). A drawback with this type of method is that an additional step is required to separate the substrate from the products, making it cumbersome for routine use. Fluorescent-labeled protein substrates such as fluorescein (EnzChek protease activity kit, Molecular Probes, Eugene, OR), fluorescein isothiocyanate (20), 2-methoxy-2,4-diphenyl-3-2H furanone, and fluorascamine (10) can also be used. The disadvantages of this are that labeling is dependent on available  $\text{NH}_2$  groups in the substrate and separation of the products from the substrate (5). These assays cannot be used in milk due to competition of milk proteins and the substrate. Birkeland et al. (1) developed a plate ELISA for *Pseudomonas fluorescens* protease with a sensitivity of 0.25 ng/ml. Another ELISA technique with an improved detection limit (0.19 ng/ml) was developed by Punj and Matta (16). An inhibition ELISA was developed by Clements et al. (4) for the detection of *P. fluorescens* protease with a detection range between

0.24 and 7.8 ng/ml. However, these assays take between 6 and 12 h and cannot distinguish between active and inactive protease. This work was undertaken to develop a rapid assay for *P. fluorescens* protease in milk that measures total as well as active enzyme.

## MATERIALS AND METHODS

### *P. fluorescens* Protease Purification

The enzyme was purified from the cell free culture supernatant of *P. fluorescens* by anion exchange chromatography (MonoQ HR 5/5; Pharmacia Biotechnology, Uppsala, Sweden) followed by size-exclusion chromatography on a Superose-12 column (Pharmacia Biotechnology). Details of the purification were described in Chapter V.

### Antibody Production

Purified *P. fluorescens* RO98 protease (Chapter V) was injected into BALB/c mice to produce polyclonal antibodies at the Utah State University Biotechnology Center (Logan, Utah). Three injections of 1 mg/ml pure protease in sterile physiological saline were given at 3-week intervals. Total serum IgG was purified from the hyperimmune serum using a protein A/G column as described in the kit instructions (Pierce Chemical Company, Rockford, IL). Antibodies were desalted and concentrated to 1 mg/ml in 0.1 M sodium phosphate buffer (pH 7.0) using a 10 kDa Centricon (Amicon Inc.) at 4,500  $\times g$  at 4°C.

### Antibody Cross-Reactivity

Seventeen strains of *Pseudomonas* were cultured in McKellar's medium aerobically at 25°C for 48 h. Cells were removed from the medium

by centrifuging cultures at  $6000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The cell-free supernatants were concentrated 10-fold using Centriprep-10 (Amicon Inc.), and resuspended in 50 mM sodium carbonate buffer (pH 8.5). The concentrates from *P. fluorescens* RO98 were adsorbed to a microtiter plate (Falcon Labware, Lincoln Park, NJ). Pure protease (1 mg/ml) and sterile medium were also adsorbed and used as positive and negative controls, respectively. The plate was blocked with 3% BSA (Sigma chemicals, St. Louis, MO) in phosphate buffered saline containing 0.025% Tween-20 (PBST; pH 7.2). The plate was washed four times with PBST between these two steps. Two hundred microliters of anti-protease antibodies (1:4000 serum dilution in PBS) was added to the wells, slowly agitated for 2 h at  $25^{\circ}\text{C}$ , and washed four times with PBST. Horse radish peroxidase (HRP) conjugated anti-whole mouse IgG (Sigma Chemical Co., St. Louis, MO) was added and incubated for 2 h, and washed four times with PBST. Activity was assayed using 3, 3', 5, 5' - Tetramethyl benzidine (Turbo TMB, Pierce Chemicals). The reaction was stopped with 2 M  $\text{H}_2\text{SO}_4$  following a 10-min incubation at room temperature. Development of yellow color was measured in an automated reflectance colorimeter (Wescor, Inc., Logan, UT).

### Immobilization of Antibodies on Beads

**Oxidation of antibodies.** Carbohydrate residues on the antibodies were oxidized using sodium *meta*-periodate (5 mg/ml) (9). The mixture was incubated at room temperature ( $25^{\circ}\text{C}$ ) in the dark for 30 min. Following the incubation, the antibodies were desalted by washing five times in a 30-kDa Centricon<sup>®</sup> (Amicon, Inc.,  $4,500 \times g$ ,  $4^{\circ}\text{C}$ ) with 0.1 M  $\text{NaPO}_4$ , (pH 7.0).

**Preparation of ceramic beads.** Ceramic beads (7 mm diameter) were

soaked in concentrated HCl for 30 min, and washed three times for 10 min with distilled water. The acid-washed beads were further incubated with 10% 3-aminopropyl-triethoxysilane (APTES, pH 4.0) (Sigma Chemicals), for 3 h at 70°C. The APTES was drained and the beads were incubated at 100°C overnight. The beads were washed with distilled water and dried at 80°C overnight and stored in a desiccator at 4°C until required (9).

***Antibody/bead conjugation.*** Poly-threonine (Sigma Chemical Co., St. Louis, MO) was covalently attached to both 2.8- $\mu$ m, tosyl-activated polystyrene Dynabeads® (Dynal, Lake Success, NY) and APTES-treated 7-mm ceramic beads in 50 mM borate buffer (pH 9.5) via the terminal amine. Four washes (three times for 10 min, and once for 30 min) with PBST (pH 7.5) were used to block remaining active sites. Adenine dihydrazine (ADH; 0.5 M in 0.1 M MES, pH 4.75; Sigma Chemical Co.) was linked to the carboxy terminal of the bound poly-threonine using an ethylene diamine carbodiimide-mediated reaction (9). Oxidized antibodies were mixed with the ADH-activated beads and incubated at room temperature for 12 h to allow crosslinking between the oxidized carbohydrate moiety of the IgG and the ADH terminal of the poly-threonine linker (9). After crosslinking, the immunomagnetic beads (IMB) were stored rotating ( $\approx$  50 rpm) in PBST with 0.02% sodium azide at 4°C until used. The ceramic beads were stored in the same conditions without rotation.

### **Immunocapture of Protease**

The immunomagnetic beads ( $3 \times 10^6$  beads) were added to 1 ml of PBS containing pure protease and allowed to rotate ( $\approx$  50 rpm) for 30 min at 25°C. Ceramic beads were used to capture added protease from skim milk because it



was not possible to completely recover all the magnetic beads from milk (2). Following capture of protease by the antibodies immobilized on the beads, IMB and ceramic beads were removed from buffer and milk, respectively. IMB were removed using a magnetic particle concentrator (DynaL MPC-E-1<sup>®</sup>, Lake Success, NY). Ceramic beads were recovered from the milk by decanting the liquid and transferring beads to a new tube. The beads were washed four times using PBST (17).

### **Captured Protease Detection**

One milliliter of secondary antibody (anti-protease,  $1.9 \times 10^{14}$ /ml) was added to the beads and incubated for 30 min at 25°C. The unbound secondary antibody was removed by washing five times with PBST. Anti-mouse IgG-HRP (1 ml,  $1.9 \times 10^{14}$ /ml) was added, and the tube was allowed to rotate ( $\approx 50$  rpm) at 25°C for 1 h. Horseradish peroxidase activity was assayed by adding 1 ml TMB (Pierce Chemicals) and the reaction was stopped by the addition of 1 ml of 2 M H<sub>2</sub>SO<sub>4</sub>. The yellow supernatant was drawn from the tubes and transferred to a 96-well microtiter plate and the color (*b*\*) was measured in an automated reflectance colorimeter (2) (Omnispec<sup>®</sup> 4000 Bioactivity Monitor, Wescor, Inc.).

### **Influence of Antibodies on Protease Activity**

The immunomagnetic beads ( $3 \times 10^6$  beads) were added to 1 ml of PBS containing pure protease (containing approximately 42 ng protein) and allowed to rotate ( $\approx 50$  rpm) for 30 min at 25°C. Beads were removed from the solution as described earlier. Following the capture of protease, IMB were washed five times with PBST and incubated with 1 ml of fluorescein labeled

casein substrate (EnzChek, Molecular Probes) at 25°C for 1 h. An equivalent amount of unbound protease was also incubated with the substrate to test the influence of antibody binding on protease activity. Increase in fluorescence, due to release of fluorescent peptides by proteolysis, was followed in a spectrofluorophotometer (RF 1501; Shimadzu, Columbia, MD) at an excitation wavelength of 480 nm and emission wavelength of 520 nm. The increase in fluorescence obtained due to hydrolysis of the substrate was expressed as RFU/h (relative fluorescence units/h). Readings obtained from a control consisting of an equal amount of IMB and substrate were subtracted from the sample reading.

#### **Influence of Antibody Concentration**

Initial trials were conducted at the paramagnetic bead and primary antibody levels used by Blake and Weimer (2). Paramagnetic beads were used at  $2 \times 10^7$  beads/ml. Antibody concentrations of 26, 2.6, 0.026, and 0.0026 ng/ml were conjugated onto the beads. The captured protease was detected in a solid phase sandwich ELISA with HRP as the reporter enzyme attached to the tertiary Ab.

#### **Sensitivity of the Assay**

Three levels of purified protease (427, 640, and 1280 ng protein) were tested in 1 ml of skim milk and PBS. Ceramic and paramagnetic beads were used in milk and buffer, respectively, to capture, concentrate, and remove the enzyme from solution. Ceramic beads were used with milk because it was not possible to completely recover the paramagnetic beads from milk. A negative control without added protease was used to adjust activity for any protease that may be present in skim milk.

## RESULTS

### Antibody Cross-Reactivity

The hyperimmune serum reacted with the pure protease from *P. fluorescens* RO98 that was used as the antigen. It also recognized protease in supernatants from 50% of the *P. fluorescens* strains and 25% of the other strains (Table 14).

### Protease Activity in the Presence of Antibodies

To determine the activity of the protease captured on the solid surface by the immobilized antibody, the beads with the captured protease were assayed with the EnzCheck kit. An increase in fluorescence (6.0 RFU/h) was observed due to release of fluorescent peptides from proteolysis with antibodies present. When an equivalent amount of protease was assayed in without antibodies, an increase of 5.4 RFU/h was obtained, suggesting the epitope for antibody binding is not the active site and that the antibody does not influence activity.

### Influence of Antibody Concentration

The solid phase capture and detection of protease was done using a sandwich ELISA with a tertiary Ab as the reporter molecule (Figure 14A). An increase in signal was observed with increasing Ab content on the bead surface (Figure 14B). This indicated that bead surface may still be available for antibody attachment because the signal did not level off.

TABLE 14. Table of cross-reactivities of murine polyclonal antibodies with *Pseudomonas* supernatants.

Microbe supernatant	Cross-reactivity
<i>P. fluorescens</i> CHA 0	+ <sup>a</sup>
<i>P. fluorescens</i> CHA 89	+
<i>P. fluorescens</i> CHA 96	+
<i>P. fluorescens</i> 3	+
<i>P. fluorescens</i> RO98	+
<i>P. fluorescens</i> B52	+
<i>P. fluorescens</i> ATCC 948	+
<i>P. fluorescens</i> 38	-
<i>P. fluorescens</i> RO13	-
<i>P. fluorescens</i> AFT 29	-
<i>P. fluorescens</i> AFT 36	-
<i>P. fluorescens</i> ATCC31732	-
<i>P. fragi</i> 71	-
<i>P. putida</i> 345	-
<i>P. xanthophilia</i> RO28	+
<i>Pseudomonas</i> spp. 113	-
<i>P. fluorescens</i> RO98 pure protease (antigen; positive control)	+
Normal mouse serum (negative control)	-

<sup>a</sup>- indicates no signal was observed in the ELISA test; + indicates a signal was observed.

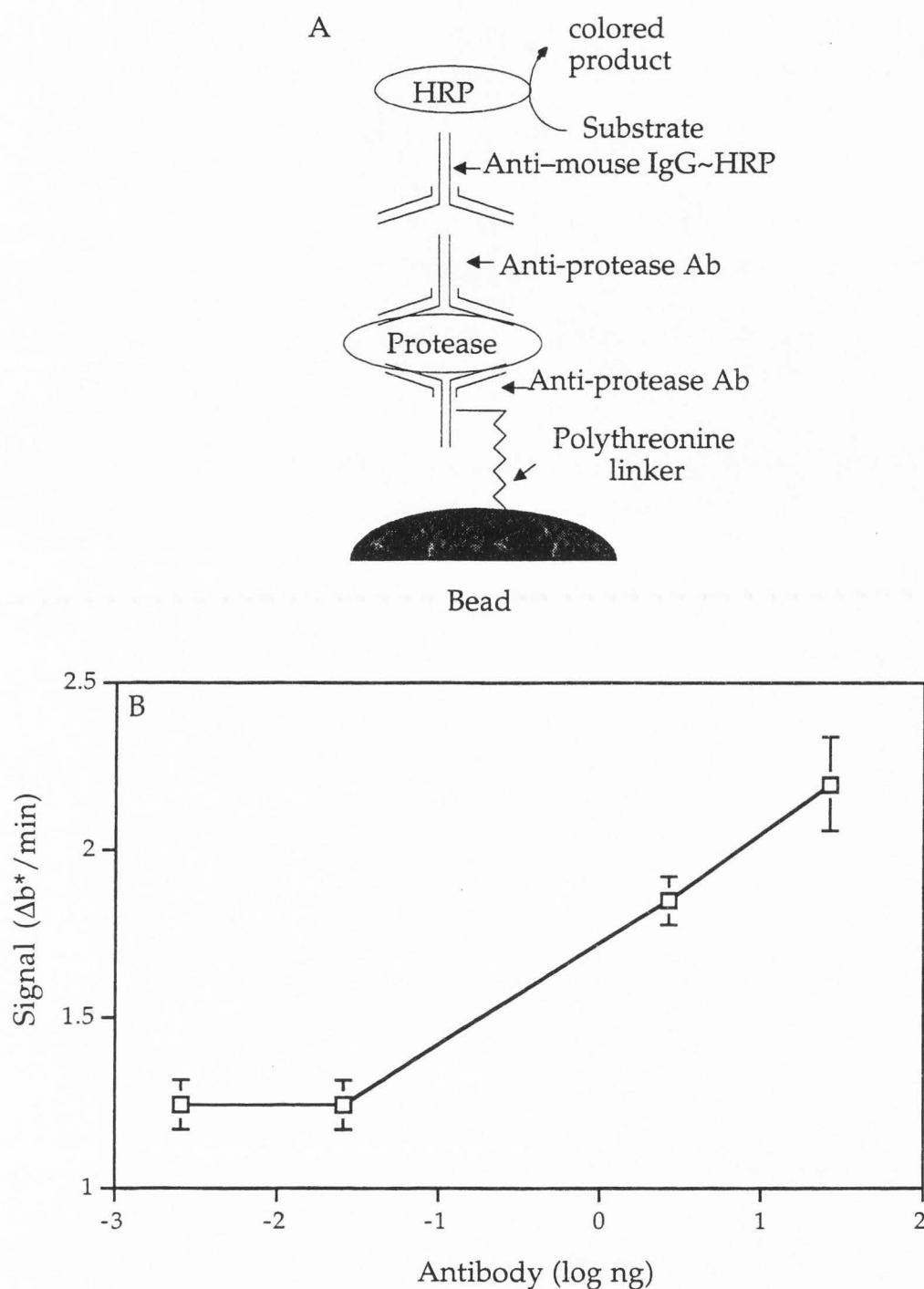


Figure 14. Detection scheme (Panel A) and influence of antibody amount (Panel B) on the capture of protease from PBS. Data points represent the mean of two replications. Error bars represent standard error of the means. Control values were used to adjust activity.

### **Influence of Matrix and Solid Surface on Enzyme Capture**

The lowest level of protease captured was 427 ng in skim milk and PBS. The assay detected all levels of enzyme added to milk and PBS in a linear relationship ( $R^2=0.99$ ) with concentration (Figure 15A, B). These levels were not intended to establish the limits of the assay, but rather to compare variation due to matrix type and differences in capture by bead type. Comparison between the matrix type is not valid due to confounding with bead type. Despite this, examination of the slopes indicated that each bead/matrix combination captured protease similarly. At each level of enzyme within each matrix the signal was significantly higher with increasing protease content.

### **DISCUSSION**

The aim of this study was to determine if a solid phase, high flow rate ELISA were feasible to capture, concentrate, remove, and detect protease from milk, thus removing the enzyme from competing substrate for further testing. To this end, polyclonal antibodies were prepared, purified, and conjugated to paramagnetic and ceramic beads, and used to capture and remove the enzyme from solution. After capturing protease from skim milk and PBS, a portion of the beads were used to detect protease activity while another was used in a sandwich ELISA to quantitate total enzyme. This approach may be industrially useful to determine the amount of active heat-stable protease in dairy products before and after processing.

The disadvantage of most protease assays in milk is that it involves either separation of proteolytic products prior to assay or competition of milk proteins with the substrate, thereby making it unsuitable for routine use in



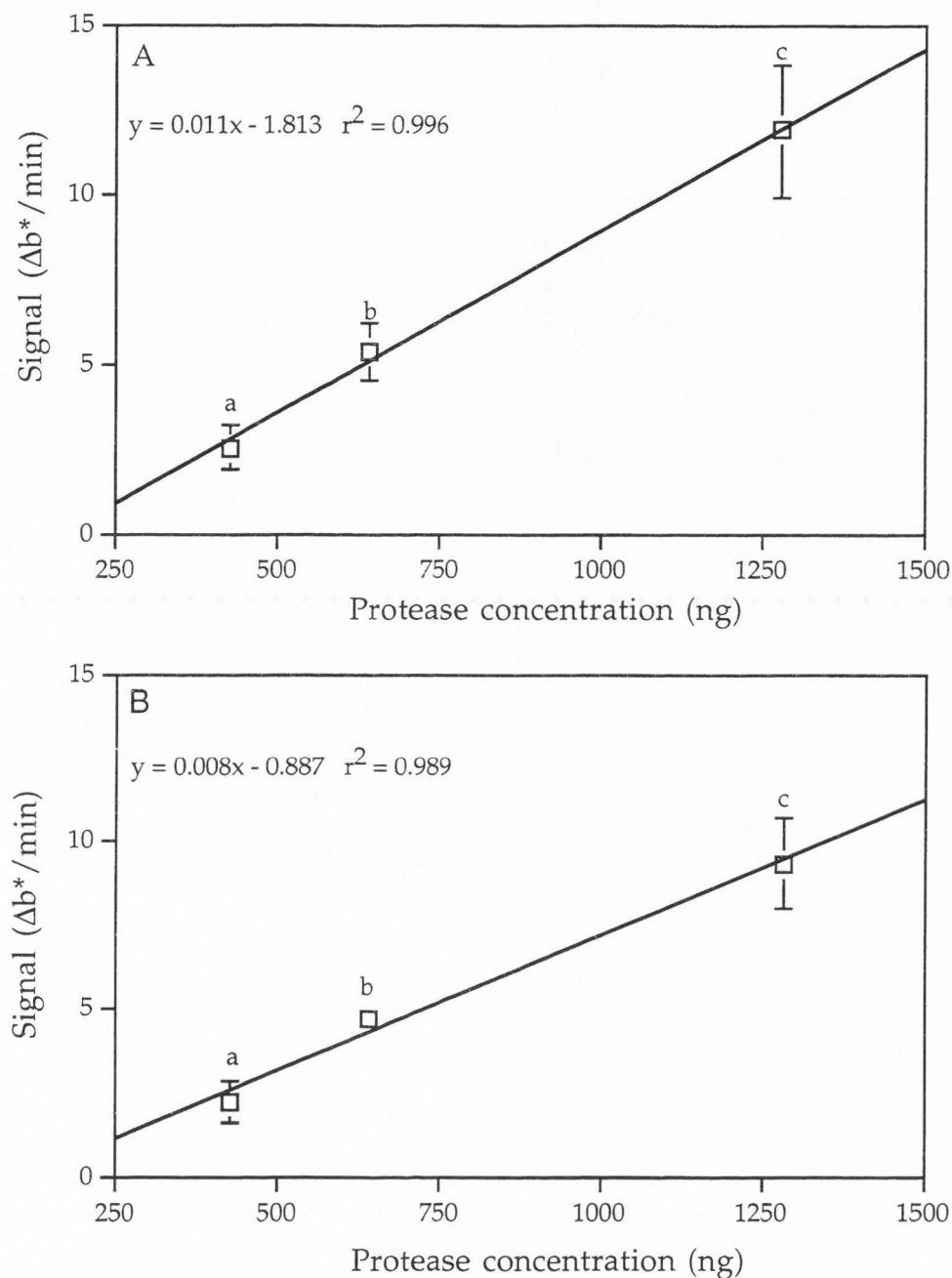


Figure 15. Influence of protease concentration on detection in PBS with immunomagnetic beads for capture and concentration (Panel A) and in milk with ceramic beads for capture and concentration (Panel B). Data points represent the mean of two replications. Error bars represent standard error of the mean. Significant differences are indicated by letters on the data point. Control assays with no protease added were used to adjust activity.

the dairy industry. As a model system, the purified antibodies from the hyperimmune serum were used to capture pure protease from PBS and milk. In an effort to design an assay that can simultaneously quantitate total enzyme and activity, the pure protease was incubated with the antibodies and protease activity was determined. No difference in protease activity was observed with or without added antibody to the protease, indicating that the active site was not the epitope and that it may be valid to use this as a strategy to determine total and active enzyme in milk.

The assay successfully captured protease in skim milk and PBS with similar signals via ELISA with two different bead types (Figure 15). This solid phase capture technique was used to separate the enzyme from the caseins by physically removing the beads from milk, thereby facilitating detection of protease. Capture of protease by the antibodies did not interfere with its activity, indicating that is feasible to use the approach in a dual phase test to determine the total protease and the active protease in milk.

The lowest level tested (427 ng/ml pure protease) was detected by this method. The lower limit of the assay may be lower than this value based on the slope of the linear fit (Figure 15). If one accepts a 1 b\* unit as the minimum signal, then the lowest detection limit would be 280 ng of protease. Previous assays using conventional sandwich ELISAs were able to detect 0.19 ng/ml (15) and 0.25 ng/ml (1). With this in mind, the sensitivity of the assay can be improved by using a spectrophotometer with TMB or by using a fluorescent substrate for HRP. Use of fluorescence usually increases the detection limit by 100 to 1000 times (11). If this type of improvement were achieved, that would bring the detection limit of this assay to 0.28 to 2.8 ng of protease. The presence of 1 ng of heat-stable protease/ml in milk prior to

UHT processing is sufficient to cause a costly reduction in shelf-life (14). The sensitivity of this assay needs to be improved so that levels that are associated with spoilage in milk can be detected. On the basis of the calculations and assumptions presented, it seems likely that this can be achieved.

The existing ELISA assays require between 6 and 12 h for a result. This time delay makes these methods less useful to the dairy industry. The assay described here is suitable for use in the dairy industry, and it can be completed in less than half the time compared to the existing methods if a static incubation were used. However, if this assay were done using high flow rate capture, the test time is reduced to 30 min (Weimer, patent pending). This can then enable diversion of raw milk containing high levels of protease to the manufacture of short shelf-life products.

Serological cross-reactivity was demonstrated between proteases produced within *Pseudomonas* species and between different strains of the same species. The antibodies cross-reacted with 50% of the 16 strains of *Pseudomonas* isolated from raw milk. Lundy et al. (13) found cross-reactivity between *P. fluorescens*, *P. lundensis*, *Pseudomonas* sp., and *P. fragi*, all of which were associated with meat spoilage. Owing to the immunological similarity of these proteases, antibodies raised to a pure *P. fluorescens* protease will detect at least some of the *P. fluorescens* proteases in milk. Additional Ab production and selection is needed to increase the range of pseudomonad proteases detected.

## CONCLUSIONS

Antibodies raised to the purified *P. fluorescens* RO98 protease cross-reacted with the proteases produced by about 50% of the isolates used in the

study within 2 h. The protease was active in the presence of the antibodies, indicating that a test can be developed that will determine the ratio of total enzyme to active enzyme after removal from milk. Protease was detected at a level of 427 ng; levels of protease lower than this need to be tested to improve the sensitivity of the assay. Further work is needed to increase the sensitivity and broaden the number of pseudomonad proteases detected.

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## CHAPTER VII

### HYDROLYSIS OF HYDROPHOBIC BITTER PEPTIDES BY PROTEASE

#### ABSTRACT

The ability of a purified protease from *Pseudomonas fluorescens* RO98 to hydrolyze bitter peptides found in Cheddar cheese was investigated. The protease was incubated with  $\alpha_{s1}$ -casein f1-9 and  $\beta$ -casein f193-209 in a model system to determine hydrolysis. Residual substrate and hydrolysis products were determined by capillary electrophoresis. Both peptides were hydrolyzed by the protease during the 90-min assay.  $\alpha_{s1}$ -Casein f1-9 was hydrolyzed into two small products and  $\beta$ -casein f193-2 degraded completely in 90 min to three other products. This protease hydrolyzed known bitter peptides that may accumulate in Cheddar cheese during aging, suggesting this protease may debitter Cheddar cheese.

#### INTRODUCTION

Bitterness is a flavor defect encountered in Cheddar and Gouda cheeses (3, 10, 16, 27). This defect is responsible for reduced acceptability and marketability of Cheddar cheese (16). Bitterness in foods may result from compounds naturally present. It may also be due to the formation of bitter compounds by chemical reactions occurring during storage (17).

Most bitter-tasting peptides in cheese originate from the hydrolysis of caseins because they contain many hydrophobic residues (18, 23). Analysis of bitter peptides formed from whole casein reveals that they originate from  $\alpha_{s1}$ - and  $\beta$ -caseins. These two caseins contain relatively high levels of amino acids with hydrophobic side groups with high hydrophobicities averaging 1.17

and 1.33 kcal/residue, respectively. Therefore, it is reasonable that they are potential sources of bitter peptides in cheese (1, 28).

Two principal factors contributing to the formation of bitter peptides in cheese are lactococcal proteases and rennet (11, 18). The proteinases produced by starter bacteria are involved in the formation of bitter peptides from  $\beta$ -casein, whereas chymosin produces bitter peptides from all the casein fractions (5, 6). Peptidases, which cleave proteins at terminal positions, may also be involved in the formation of bitterness as they break down the larger non-bitter fragments generated by proteases into bitter peptides (25, 26).

Technological advances for the acceleration of cheese ripening, low-fat cheese development, and production of altered milk components using recombinant DNA techniques often results in increased bitterness in cheese. This highlights the need to avoid the potential defect of bitterness in cheese (8, 17).

Proteolytic enzymes can be added directly to cheese curd (8, 9). Addition of microbial acid protease results in increased bitterness, but by mixing different peptidases and proteases, a more desirable cheese flavor with reduced bitterness can be obtained (13, 21). Edam cheese was debittered using peptidases from *Pseudomonas fluorescens* VTTE 8.7 (19). The same enzymes were able to prevent bitterness and accelerate ripening in Cheddar cheese made with calf rennet (20). The objective of this study was to determine the ability of a purified protease from *P. fluorescens* RO98 to hydrolyze bitter peptides associated with bitterness in Cheddar cheese.

## MATERIALS AND METHODS

### Protease Purification

The enzyme was purified from the cell free culture supernatant of *P. fluorescens* by anion exchange chromatography (MonoQ HR 5/5; Pharmacia Biotechnology, Uppsala, Sweden) followed by size-exclusion chromatography on a Superose-12 column (Pharmacia Biotechnology). Details of the purification were described in Chapter V.

### Substrate Synthesis

Two peptides (Table 15) associated with bitterness in Cheddar cheese (1, 17, 28) were synthesized at the Utah State University Biotechnology Center. Individual amino acids (Perseptive Biosystems, Foster City, CA) were activated by PyAOP (7-Azabenzotriazol-1-yloxytris (pyrrolidino)phosphonium-hexafluorophosphate). The peptide was synthesized using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry on a

TABLE 15. Bitter peptides used in this study.

Origin	Sequence	Hydrophobicity	Molecular weight	Reference
$\alpha_{s1}$ -casein f(1-9)	RPKHPIKHQ	1422	1143	1
$\beta$ -casein f(193- 209)	YQQPVLG PVRGPFPIIV	1762.4	1881	28

polyethelene glycol-polystyrene resin as a solid support using a PE-Biosystems 9050 peptide synthesizer (Perseptive Biosystems). The synthetic peptide was cleaved from the resin by trifluoroacetic acid (TFA) (Fisher Scientific, Pittsburg, PA). The peptide was precipitated in ether and lyophilized in a benchtop lyophilizer (Virtis, Gardiner, NY).

### Substrate Purification

The synthetic peptides were purified by preparative reverse-phase HPLC on a C8 Brownlee Aquapore RP-300 column (4.6 mm x 10 cm Perkin-Elmer/Applied Biosystems, Foster City, CA). The two eluants employed were; 0.1% (v/v) trifluoroacetic acid in distilled water (A) and, 0.085% trifluoroacetic acid in 80% acetonitrile (B). The peptides were resolved using a gradient of eluant B in eluant A from 0 to 45% in 45 min, at a flow rate of 1 ml/min and detected by continuously monitoring the absorbance of the eluate at 200 nm. Pure peptide fractions were lyophilized and stored at -20°C until required.

### Peptide Hydrolysis

The reaction mix consisted of 1 mg/ml of each peptide substrate, 256 µg/ml purified *P. fluorescens* RO98 protease (50 µl; Chapter V) in Jenness-Koops buffer (pH 6.8) (12). The reaction incubated for 0, 15, 30, 60, and 90 min at 30°C. Enzyme activity was stopped with the addition of 100 µl of 100 mM sodium citrate (pH 2.8). Two controls, one consisting of peptide only and the other consisting of enzyme only, were used to determine spontaneous degradation of the peptide and enzyme, respectively. Samples were centrifuged at 16,000 x g for 10 min at 4°C. The clear supernatants were removed and filtered through a 0.2-µm low protein binding syringe filter

(Amicon Inc., Beverly, MA) and stored at  $-70^{\circ}\text{C}$  for analysis with capillary electrophoresis (CE).

### Analysis of Peptides

Capillary electrophoresis was done as described by de Jong et al. (4) with the following modifications. Urea concentration was reduced from 6 to 4 M and 0.1% hydroxypropyl methyl cellulose (HPMC) was used as the polymeric additive instead of 0.05% methylhydroxyethyl cellulose. Electrophoresis run buffer consisted of 4 M urea, 20 mM citrate, 10 mM phosphate and 0.01% HPMC (pH 3.30). The sample was diluted in sample buffer (pH 3.95) to a final concentration of 4 M urea and 2.5 mM citric acid. Samples were electrophoresed on a 75  $\mu\text{m}$   $\times$  57 cm coated capillary (P1, Supelco, Bellefonte, PA) at 316 volts/cm (18 kV, 23 microamps at  $38^{\circ}\text{C}$ ). The run time was 10 and 20 min for the  $\alpha_{s1}$ -casein f1-9 and  $\beta$ -casein f193-209 peptides, respectively.

## RESULTS

### Hydrolysis of $\alpha_{s1}$ -Casein f1-9

The pure enzyme was incubated with  $\alpha_{s1}$ -casein f1-9 to examine its ability to hydrolyze this bitter peptide. The elution times for the peaks shifted slightly between the runs, which is known to occur depending on the conditioning of the column (1, 24), but this did not hinder interpretation of the results. Nearly 50% of the substrate, which eluted at  $\sim 6.2$  min, was degraded after 90 min of incubation, resulting in two new products. A new peptide eluted at 7.1 min within 15 min of incubation (Figure 16A). Another second hydrolysis product eluted at 6.6 min after 30 min of incubation. Both

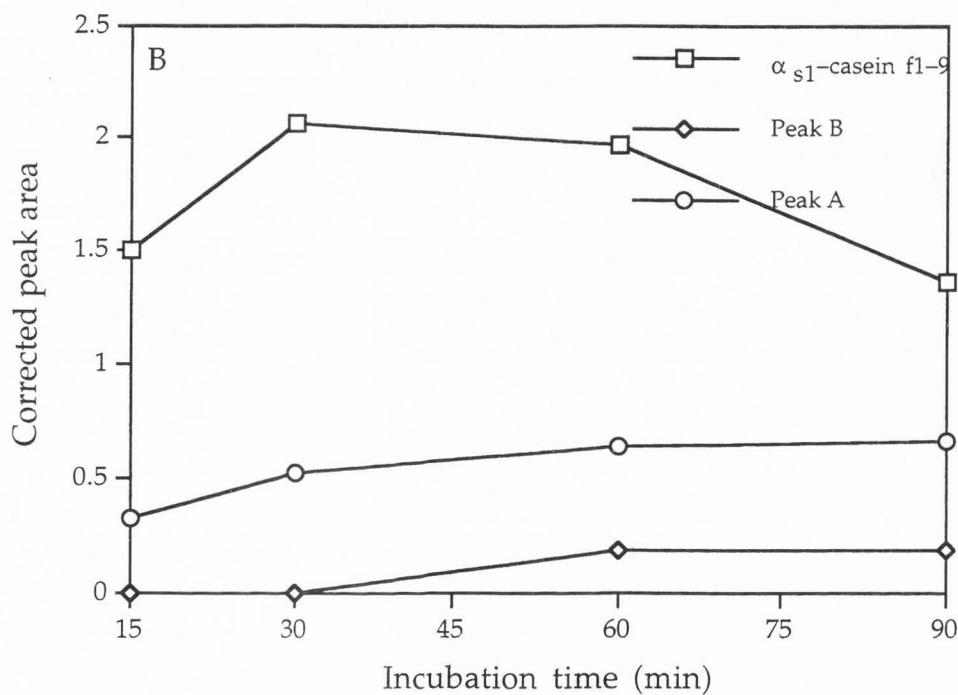
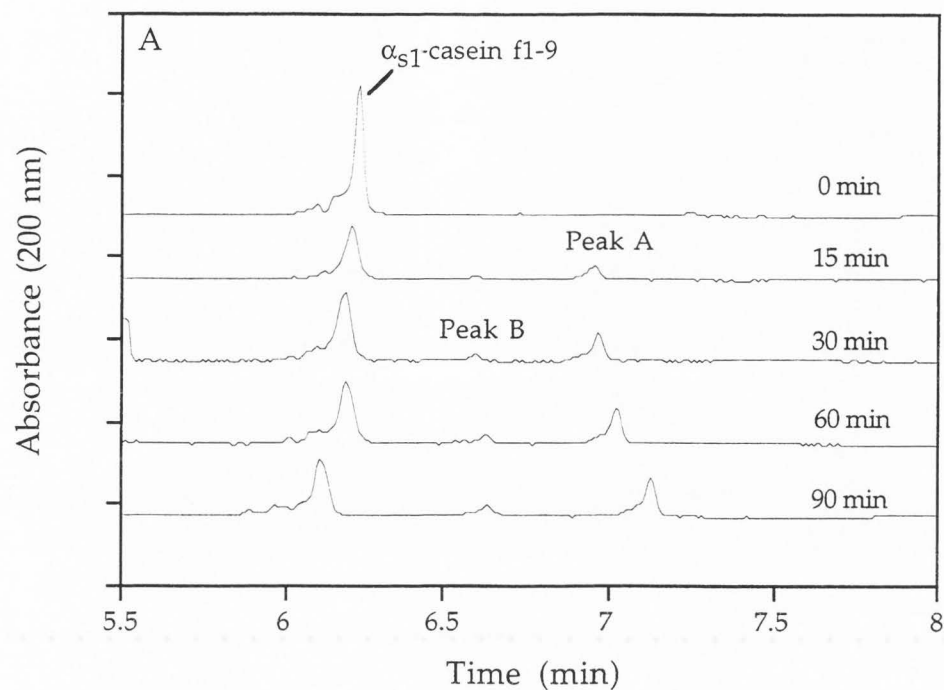


Figure 16. Electropherograms showing hydrolysis of  $\alpha_{s1}$ -casein f1-9 by *P. fluorescens* protease (Panel A). The change in peak area of the substrate and products over time is shown in Panel B;  $\alpha_{s1}$ -casein f1-9 (—○—), peak A (— —), and peak B (—◇—).



products increased initially, but remained constant by 45 min of incubation (Figure 16B).

### Hydrolysis of $\beta$ -Casein f193-209

The enzyme also hydrolyzed another known bitter peptide,  $\beta$ -casein f193-209, during the assay. The substrate eluted at ~16.5 min and was hydrolyzed completely after 90 min (Figure 17A). New peptides eluting at 13.1 min (Peak A) and 9.2 min were the initial products of hydrolysis. Peak A broadened at 90 min and a third peak appeared (Peak C), suggesting degradation of the peptide in Peak A into this new peak. A peak eluting at 11.3 min (peak C) was  $\beta$ -casein f193-209 was available. The rates of hydrolysis and product appearance were different between the products (Figure 17B). Peak B initially increased, but remained constant during the assay time. Peak C appeared only after 90 min. The substrate was completely hydrolyzed by 90 min.

## DISCUSSION

The aim of this study was to determine the ability of a purified protease from *P. fluorescens* to debitter cheese by hydrolyzing peptides associated with bitterness in Cheddar and Gouda cheese (1). Bitter peptides are produced by chymosin and the starter culture bacteria that are added during cheese making (18, 25). Ripening results in various amounts of these peptides because starters and non-starter bacteria have different proteolytic characteristics (17). An additional explanation may be associated with the residual heat-stable protease produced by psychrotrophic bacteria that are commonly found contaminating raw milk (2). These bacteria grow during

refrigeration of raw milk and produce proteolytic enzymes that resist pasteurization (2, 7, 22, Chapters IV, V). Therefore, cheese made from milk containing psychrotrophic bacteria may contain heat-stable protease, which remains active at the temperature and pH of cheese matrix during aging (Chapter V).

Both the peptides associated with bitterness were hydrolyzed by the purified *P. fluorescens* protease. Two products were formed from  $\alpha_{s1}$  f1-9 (Figure 16), and the substrate was reduced by 50% during the assay.

Assessment of the bitterness of the new peptides was beyond the scope of this work, but it is possible that the new products were bitter as well. However, the  $\alpha_{s1}$  f1-9 fragment is not as bitter as  $\beta$ -casein f193-209 (17). The bitter flavor recognition level for  $\alpha_{s1}$ -casein f1-9 is 0.78 mg/ml in water (pers. comm., Charlotte Brennand, September, 1998). The purified protease hydrolyzed about 50% of the peptide at the end of the 90-min incubation period. The estimated impact of this enzyme on reducing bitterness is 50% reduction in bitterness caused by this peptide.

$\beta$ -casein f193-209 has a bitter flavor recognition level of 0.35 mg/ml (pers. comm., Charlotte Brennand, September, 1998), and 1 mg of this peptide was incubated with the purified protease. Theoretically, this protease can completely eliminate bitterness due to this peptide because the peptide was completely hydrolyzed during the 90-min incubation period. However, the flavor profile of products is unknown. Three products formed from  $\beta$ -casein f193-209, all of which eluted before the substrate indicating they are less hydrophobic than  $\beta$ -casein f193-209 (Figure 17). The  $\beta$ -casein f193-209 substrate was completely degraded by 90 min, and at the same time Peak A began to broaden, suggesting the contents of this peak were being used as a

new substrate after  $\beta$ -casein f193-209 was depleted. A new peak appeared (Peak C) supporting this assumption. Based on the bitterness threshold and substrate concentration, one can hypothesize that this enzyme will impact the bitter defect in cheese.

Identification and evaluation of bitterness of the peptides resulting from each substrate were beyond the scope of this study. Further work is needed to assess the impact of this enzyme in reducing bitterness in cheese. However, some precedent exists in the literature that this approach will result in cheese with less bitterness. Law (14) added a preparation containing proteinase-peptidase from *Pseudomonas* to cheese curd during salting of Cheddar cheese. Low levels of added enzyme accelerated as well as enhanced ripening while large amounts caused bitterness. Similarly, addition of neutrase, a neutral proteinase from *Bacillus subtilis* also enhanced flavor of Cheddar cheese at low levels of addition (15). These studies suggest that pseudomonad proteases, when used in appropriate amounts, can reduce bitterness in cheese.

## CONCLUSIONS

The protease hydrolyzed the two hydrophobic peptides associated with bitterness in Cheddar cheese. The  $\alpha_{s1}$ -casein f1-9 was reduced by 50% during the 90 min incubation and all the  $\beta$ -casein f193-209 peptide was converted to other hydrolytic products. The results indicate that the protease has the potential to debitter Cheddar cheese as it shows the ability to hydrolyze hydrophobic peptides generated by rennet and starter enzymes. The ability of the protease to hydrolyze hydrophobic peptides preferentially in the presence of less hydrophobic peptides needs to be addressed in the future.

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CHAPTER VIII  
INFLUENCE OF GROWTH CONDITIONS ON LIPASE  
ACTIVITY IN *PSEUDOMONAS*<sup>1</sup>

ABSTRACT

Production of multiple lipases in various species of *Pseudomonas* isolated from raw milk was investigated. Each strain was studied for production of lipase in four media during a 50-h incubation period. Lipase activity varied significantly ( $P < 0.001$  to  $0.003$ ) among the strains, growth media, and incubation times tested. Lipase activity of the strains varied significantly ( $P < 0.001$ ) with the growth medium. Pasteurization resistance of lipase activity was also significantly ( $P < 0.02$ ) influenced by stage of growth. Cross-reactivity with monoclonal antibodies made against a commercial *Pseudomonas fluorescens* lipase was tested with supernatants from 16 *Pseudomonas* strains. The culture supernatant of *P. fluorescens* 31732 reacted with all the monoclonal antibody clones tested except one. Other strains, except *P. fluorescens* P3, demonstrated limited reactivity. Zymograms of *Pseudomonas fragi* 71 and *P. fluorescens* RO98 supernatants yielded four different bands of activity. Three peaks with lipase activity were purified from the culture supernatant of a raw milk isolate, *P. fragi* 71. Electrophoretic mobility of each lipase peak revealed that the lipase preparations contained a different protein associated with extracellular lipase activity. Multiple lipases may be produced by pseudomonads.

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<sup>1</sup> Coauthored by Ramarathna Koka and Bart Weimer



## INTRODUCTION

The widespread use of refrigeration on farms and in milk processing plants improves milk quality by inhibiting the growth of many mesophilic and coliform bacteria (17), but selects for the growth of psychrotrophic bacteria. *Pseudomonas* sp. predominate in milk stored at low temperatures before pasteurization. Additionally, these bacteria are common post-processing contaminants that grow and produce more spoilage enzymes in the finished product during storage (2). The predominant lipolytic species in raw milk are fluorescent pseudomonads (37).

Lipases adsorb onto the surface of the milk fat globule membrane and are, therefore, concentrated in butter and cheese. Unlike proteases, they are not lost in the whey portion of fermented milk products. As a result of this property, residual lipase activity poses a problem in long shelf-life dairy products including cheese, butter, and ultra high temperature treated (UHT) fluid milk (1, 10, 12, 14, 15, 26). Consequently, the growth and production of heat-stable enzymes by psychrotrophic bacteria are problems in products intended for distant markets because they remain active after pasteurization and UHT processing of fluid milk (8). Comparison of heat stabilities of lipases reported in different studies is difficult because residual activity depends on methodology, heating media used, degree of purification of the enzyme, and the strains tested (36). Increased knowledge regarding enzyme production by pseudomonads in milk will lead to control strategies that decrease enzyme activity in raw milk. Hence, processed milk products will contain fewer spoilage enzymes and presumably less degradation and associated off-flavors leading to less spoilage in long shelf-life dairy products.

Strain variability intensifies the problem of lipolytic spoilage in milk

and makes lipase detection and prevention of spoilage in long shelf-life dairy products difficult. *P. fragi* NRRL B-25 produces two bands of activity on a zymogram, which separate into a light and a heavy component upon resolution using gradient ultracentrifugation, the heavier enzyme being an aggregate of the lighter one (30). Similar reports are available for *P. fragi* NCDO 752 and *Micrococcus freundenreichii* NCDO 1223, which produces two lipolytic peaks that are determined to be multimeric forms of the same enzyme (27).

Distinctly different lipases may be produced in *Pseudomonas*. Information on the number of lipases produced by pseudomonads and the environmental parameters that regulate their production is not currently available. Lack of information on diversity of lipases from *Pseudomonas* prompted this study to determine the influence of environmental factors on lipase production.

## MATERIALS AND METHODS

### Strains and Media

Seventeen isolates of *Pseudomonas* were used in this study (Table 16). Of the 17 strains, nine strains were raw milk isolates (9, 13). Working cultures were prepared from frozen culture stocks stored at  $-70^{\circ}\text{C}$  in reconstituted nonfat dry milk containing 20% glycerol. An inoculum was freshly prepared from a 24-h culture (0.2 optical density) by harvesting the cells with centrifugation at  $6000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The inoculum was washed three times with sterile saline and resuspended in sterile saline to an  $\text{OD}_{620}$  of 0.2. This was used to inoculate each sterile medium. The media used in this study were nutrient broth (Difco Laboratories, Detroit, MI), McKellar's

TABLE 16. Strains used to study lipase.

Organism	Strain name	Code	Source/ reference	Comments
<i>P. fluorescens</i>	38	1	9	milk spoilage
<i>P. fluorescens</i>	CHA96	2	25	GacA <sup>-</sup> /pME3066
<i>P. fluorescens</i>	CHA0	3	25	GacA <sup>+</sup> , wildtype
<i>P. fluorescens</i>	CHA89	4	25	GacA <sup>-</sup> , mutant
<i>P. fluorescens</i>	3	8	9	milk isolate
<i>P. fluorescens</i>	RO98	9	13	raw milk isolate
<i>P. fluorescens</i>	B52	11	35	raw milk isolate
<i>P. fluorescens</i>	RO13	12	13	raw milk isolate
<i>P. fluorescens</i>	AFT29	13	18	
<i>P. fluorescens</i>	AFT36	14	18	
<i>P. fluorescens</i>	948	15	ATCC	
<i>P. fluorescens</i>	31732	16	ATCC	
<i>P. fluorescens</i>	LS107d2	17	23	
<i>P. fragi</i>	71	5	9	milk spoilage
<i>P. putida</i>	345	7	9	milk spoilage
<i>Pseudomonas</i> sp.	113	6	9	milk spoilage
<i>P. xanthophilia</i>	RO28	10	13	raw milk isolate

minimal medium with lipase inducer (29), UHT skim milk (Gossner Foods, Logan, UT), and Chrisope's medium (6). Composition of the media is listed in Table 33 (Appendix). Inoculated media were incubated at 10 ( 2°C with shaking at 200 rpm in a 500-ml flask.

Lipase inducer for McKellar's medium was prepared from UHT skim milk by acidification with 1 N HCl to pH 4.6 to precipitate the caseins followed by centrifugation (7000 × g for 10 min at 4°C), and filtration through a Whatman #1 filter paper (Whatman International Ltd., Maidstone, UK). The filtered whey was neutralized to pH 7.0 using 1N HCl and filter-sterilized through a 0.22-μm syringe filter (Gelman Sciences Inc., Ann Arbor, MI), and

stored at  $-70^{\circ}\text{C}$  until required. This was added (1% v/v) to sterile McKellar's minimal medium.

### Sampling

Samples (10 ml) were drawn from each medium at 24 and 50 h and divided into two subsamples. One half was heat treated at  $62.5 \pm 0.5^{\circ}\text{C}$  for 30 min in a water bath, and the other half was not given a heat treatment. These two subsamples were subsequently referred to as heated and not-heated, respectively. The measurements were done in duplicate.

### Total Plate Counts

Total plate counts were determined at both 24 and 50 h before heat treatment of the culture using the standard spread plate method (33).

### Lipase Assay

Lipolytic activity was assayed by reflectance colorimetry as described by Blake et al. (4) at 24 and 50 h of incubation in both heated and not-heated samples using p-nitrophenyl caprylate in a 96-well microtiter plate. Development of a yellow color due to the hydrolysis of the chromogenic substrate was monitored for 3 h at  $37^{\circ}\text{C}$  in a reflectance colorimeter (Omnispec 4000 Bioactivity Monitor; Wescor, Inc., Logan, UT). The data were analyzed as described by Dias and Weimer (11). Plate counts were used to calculate activity obtained per cell (Eq. 1). Enzyme activity was expressed as  $\Delta b^*/h$ . All assays were performed in duplicate and reported as mean values. Thermostable lipase activity was measured as residual activity following heat treatment.

$$\text{Activity } (\Delta b^*/h) = (\text{Average activity} / \text{Average plate count}) \times 10^8 \quad [\text{Eq. 1}]$$

### Quantitation of Protein

Protein concentration was monitored continuously by measuring absorbance at 280 nm during chromatography (System Gold, Beckman Instruments, CA). Protein concentration was determined using a BCA protein assay kit for all other determinations (Pierce Chemical Company, Rockford, IL). A standard curve was constructed using BSA.

### Culture Growth for Lipase Production

The inoculum was prepared from a 24-h culture of *P. fragi* 71 as described earlier and inoculated (1% of a suspension standardized to  $A_{620} = 0.2$ ) into 14 L of sterile McKellar's medium with 1% (v/v) lipase inducer (29). The vessel was incubated aerobically at 4°C for 48 h by constantly purging filtered air through a 0.22  $\mu\text{m}$  filter (Amicon, Inc., Beverly, MA). The cells were removed from the culture medium by centrifugation ( $6000 \times g$  for 10 min at 4°C), and the supernatant was concentrated to 500 ml by ultrafiltration through a 10-kDa membrane in a tangential flow ultrafiltration system (Millipore Corporation, Bedford, MA). This was further concentrated to 10 ml in a stirred cell ultrafiltration unit with a 10-kDa membrane (Amicon Inc.). The resulting concentrate was filter-sterilized through a 0.22- $\mu\text{m}$  syringe filter and stored at -70°C until further use. The cell-free concentrate was applied to a Mono Q column (HR 5/5) (Pharmacia Biotechnology, Uppsala, Sweden) and eluted with a linear salt-gradient of 1 M NaCl in 20 mM sodium phosphate buffer (pH 6.2) at a flow rate of 1 ml/min. Fractions were collected at 1-min intervals and individually desalted using five column volumes of 20 mM sodium phosphate buffer (pH 6.2) in 10-kDa Centricon ultrafiltration units (Amicon Inc.). Peaks were collected and assayed for lipase activity, and its purity was checked by SDS-PAGE.

### Electrophoresis and Electroblotting

SDS-PAGE was conducted in reducing conditions as described below (24). A discontinuous polyacrylamide gel (160 x 175 x 0.75 mm) with 5% stacking (pH 6.8) and 14% resolving (pH 8.8) gel was used. The gel was run in a Protean II Xi electrophoresis cell (BioRad Laboratories, Hercules, CA) for 3 h at a constant current of 30 mA. Following electrophoresis, the proteins were blotted to a polyvinylidene fluoride membrane (Immobilon-PSQ; Millipore Corporation) overnight in a Trans-blot cell (BioRad Laboratories) at a constant voltage of 14 V.

### Urea-PAGE and Zymogram

Lipolytic activity of the culture supernatants was examined by activity staining. Urea-PAGE was conducted as described by Yiadom-Farkye (39) in a Protean II Xi electrophoresis cell (Biorad Laboratories). The stacking gel consisted of 0.05 M Tris (pH 6.8), 7 M urea, and 5% acrylamide with N, N'-methylene-bis-acrylamide (Sigma Chemical Co., St. Louis, MO). The resolving gel was made of 0.3 M Tris (pH 8.8), 7 M urea, and 7% acrylamide with N, N'-methylene-bis-acrylamide. Reservoir buffer contained 0.025 M Tris and 0.019 M glycine. The lipase preparation (22  $\mu$ l) was mixed with an equal volume of sample buffer (8 M urea in stacking gel buffer) to which  $\beta$ -mercaptoethanol (3.2%) and 1  $\mu$ l tracking dye (1% bromophenol blue in 10% sucrose) were added. Samples were placed in a 40°C water bath for 30 min prior to electrophoresis. The gel was run at a constant current of 20 mA through the stacking gel and 30 mA through the resolving gel for 3 h. Following electrophoresis, the proteins were blotted onto a polyvinylidene fluoride membrane (Immobilon-PSQ; Millipore Corporation) in a trans-blot cell and stained for lipase activity using the method of Benoist and



Schwencke (3). The blot was equilibrated for 10 min in 100 ml buffer (100 mM HEPES pH 7.5, 100 mM NaCl, 6 mM  $\text{CaCl}_2$ ), in the dark at room temperature. Next, the blot was placed in an activity stain containing 1 mM *p*-nitrophenyl caprylate in 50 mM MES/KOH; 50 mM HEPES/KOH (pH 7.5), 100 mM NaCl, and 5 mM  $\text{CaCl}_2$ . Lipase activity was detected as yellow bands within 5 min.

#### **Serological Cross-Reactivity Among Lipases**

*P. fluorescens* lipase (Fluka, Buchs, Switzerland) was injected into BALB/c mice to produce monoclonal antibodies at the Utah State University Biotechnology Center. Total serum IgG was purified using a protein A/G column as described in the kit instructions (Pierce Chemicals, Rockford, IL). Antibodies were desalted and concentrated to 1 mg/ml in 0.1 M sodium phosphate buffer, (pH 7.0) using a 10 kDa-Centricon (Amicon Inc.) at 4,500  $\times g$  at 4°C.

The purified antibodies were used to screen for lipase in culture. Cell-free supernatants from 48 h cultures were concentrated 10 fold using Centriprep-10 (Amicon Inc.) and resuspended in 50 mM sodium carbonate buffer (pH 8.5). The concentrate was adsorbed to a microtiter plate (Falcon Labware, Lincoln Park, NJ) overnight at 4°C, and blocked with 3% BSA in phosphate buffered saline containing 0.025% Tween-20 (PBST; pH 7.2). The plate was washed four times with PBST in between these two steps. Three hundred microliters of anti-lipase antibodies (1:4000 serum dilution in PBS) was added to the wells, slowly agitated for 2 h at 25°C, and washed four times with PBST. Horse radish peroxidase (HRP) labeled anti-whole mouse IgG (Sigma Chemical Co.) was added to label anti-lipase antibodies for 2 h, then washed four times with PBST. Activity was assayed using 3, 3', 5, 5' -

Tetramethyl benzidine (1-Step Turbo TMB®, Pierce Chemicals). The reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub>. Development of yellow color was measured in an automated reflectance colorimeter.

### Statistical Analyses

Data were analyzed by analysis of variance using the Minitab statistical software (Release 9.1, Minitab, Inc., State College, PA) in a 17 × 4 × 2 × 2 (Eq. 2) factorial with strain, medium, incubation time, and heat treatment as the main effects. The three-way and four-way interaction mean squares were pooled to obtain the error term (Eq. 3) because no replication was done (pers. comm., Dr. D. Sisson, July, 1998). Least square difference (32) tests were used to calculate the differences between means ( $\alpha < 0.05$ ).

$$Y_{ijklm} = \mu + S_i + M_j + H_l + Sm_{ij} + T_k + St_{ik} + Mt_{jk} + Th_{kl} + SH_{il} + MH_{jl} \quad [\text{Eq. 2}]$$

$$\text{Error} = SMT_{ijk} + SMH_{ijl} + STH_{ikl} + MTH_{jkl} + SMTH_{ijkl} + d_{ijklm} \quad [\text{Eq. 3}]$$

S = strain; M = Medium; T = incubation time;

H = heat treatment; Y = phospholipase activity, d = duplication

## RESULTS

### Influence of Medium, Strain, Incubation Time, and Heat Treatment

Growth in different media significantly influenced ( $P < 0.001$ , Table 17) production of lipase activity. The maximum total activity was produced in nutrient broth (Figure 18A). Lipase was not observed in McKellar's medium in the absence of the inducer, which consisted of whey proteins, however, activity was restored with addition of this component. No significant

TABLE 17. ANOVA for lipase production.

Treatment	df	P-value
Medium (M)	3	<0.001
Strain (S)	16	0.003
Incubation time (I)	1	<0.001
Treatment (T)	1	<0.001
M × S	48	<0.001
M × I	3	0.1755
M × T	3	0.2036
S × I	16	<0.001
S × T	16	0.378
I × T	1	0.024
Error <sup>a</sup>	163	—

<sup>a</sup> Error term was the sum of duplication, three-way and four-way interactions

difference in activity was observed between skim milk and McKellar's medium.

Strains produced significantly ( $P < 0.003$ ) different amounts of lipase activity, ranging from 2.4 to 11.3  $\Delta b^*/h$  (Figure 18B). Among the *P. fluorescens* isolates tested, ATCC 948 (code 15) produced the most activity and LS107d2 (code 17) produced the least. Among the other species tested, *P. fragi* 71 (code 5), a raw milk isolate, produced the most activity while *Pseudomonas* spp.113 (code 6), another raw milk isolate, produced the least (Figure 18C). Pasteurization treatment significantly decreased the activity of lipase ( $P < 0.001$ ; Table 17). Residual activity was 69% of the unheated sample (Figure 18D).

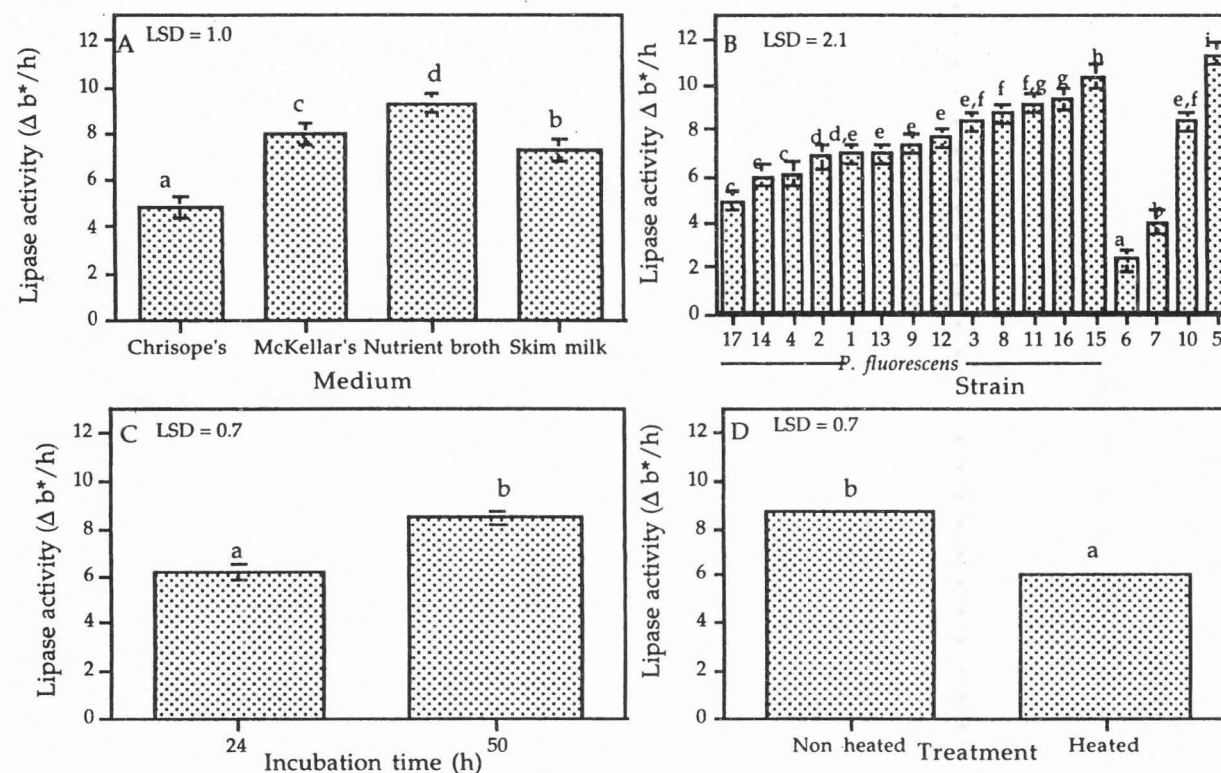


Figure 18. Influence of growth conditions on lipase activity. Panel A shows the influence of growth medium, panel B shows the influence of strain (Strain names are provided in Table 16), panel C shows the influence of incubation time, and panel D shows the influence of heat treatment (62.5°C). Activity has been summed over the other three effects for each main effect. Error bars represent standard error of the mean. In some figures the error bar may not show due to the small magnitude. The alphabets represent least significant difference between the means. The same letter on a bar indicates no significant difference.

### Treatment Interactions and Lipase Activity

Strains grown in different media (medium x strain) produced significantly different ( $P < 0.001$ ) amounts of lipase activity (Figure 19A). Forty percent of the strains produced maximum lipase activity when grown in skim milk compared to growth in the other media. Maximum activity was observed in *P. xanthophilia* RO28 (code 10) grown in McKellar's medium (Figure 19A).

Length of incubation significantly ( $P < 0.001$ ) increased lipase activity of the strains tested (incubation time x strains). All the *P. fluorescens* strains (codes 1 to 4, 8, 9, 11 to 17) produced higher lipase activity after 50 h of incubation than at 24 h (Figure 19B). The converse was true for the other species tested. The influence of heat treatment on the lipase activity among strains was not significant ( $P = 0.378$ , Figure 32A, Appendix). Incubation for 50 h produced significantly ( $P < 0.02$ ) more heat-stable lipase activity than growth for 24 h (Figure 19C).

Heat stability of lipase was not significantly influenced by the medium of growth ( $P = 0.2036$ , Figure 32C, Appendix). More lipase activity was observed in all the media after 50 h of growth, although it was not statistically significant ( $P = 0.1755$ ) (Figure 32B, Appendix).

### Enzyme Diversity

To further examine the lipase content of the supernatant, serological relatedness, zymogram analysis, and partial purification were done. Immunological relatedness of the lipase was tested by reacting culture supernatants of the strains with antibodies from various monoclonal hybridomas produced to a commercial *P. fluorescens* lipase.

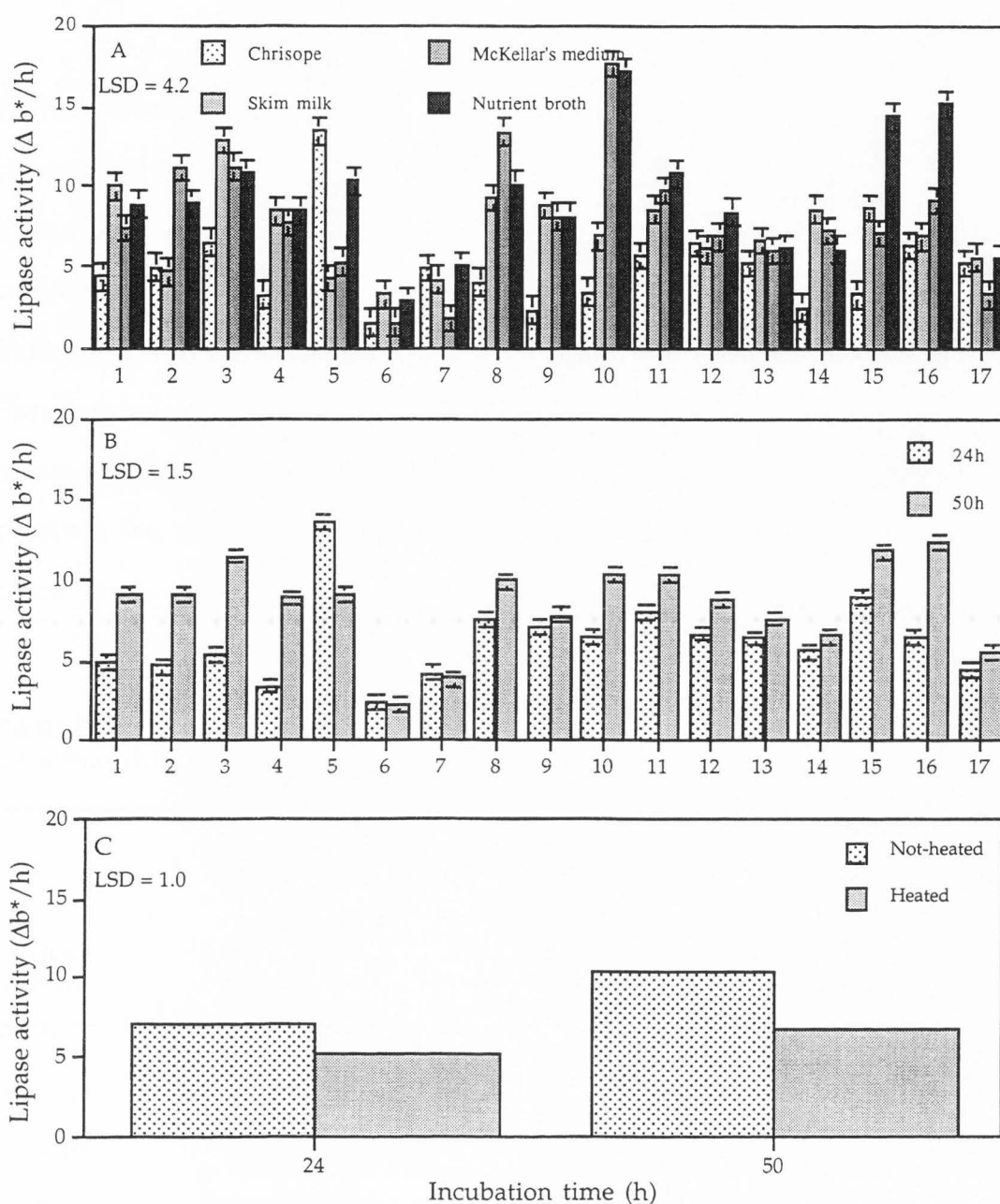


Figure 19. Influence of growth conditions on lipase activity. Panel A shows the influence of strain  $\times$  medium, panel B shows the influence of strain  $\times$  incubation time, and panel C shows the influence of incubation time  $\times$  heat treatment. Strain numbers correspond to the strain names in Table 16. Error bars represent standard error of mean.

**Serological relatedness.** Eight hybridomas to a commercial lipase from *P. fluorescens* (Fluka) were used to test the immunological relatedness of the lipase produced by the isolates and to select a clone for future use in a rapid lipase test. Strains 71, CHA96, CHA0, CHA89, 113, LS107d2, and B52 did not cross-react with any clone tested. *Pseudomonas putida* 345 (code 7) and *P. fluorescens* RO13 (code 12) reacted with the same clones (Table 18). *Pseudomonas fluorescens* ATCC31732 (code 16) was similar to the commercial lipase, leading to the speculation that this strain was used to produce the enzyme commercially. All but one monoclonal antibody (MAb) clone produced against commercial *P. fluorescens* lipase cross-reacted with

TABLE 18. Reactivity of hybridoma clones to crude cell-free culture supernatant of *Pseudomonas* strains.

MAb clone	Strain supernatants <sup>b</sup>								
	P3	P38	P345	AFT 29	RO13	RO28	RO98	ATCC 31732	Fluka lipase
A3.8	+ <sup>c</sup>	-	-	-	-	-	-	-	+
A3.17.4	+	-	-	-	-	+	-	+	+
C5.1.18	-	-	+	-	+	-	+	+	+
C5.4.15	-	-	-	-	-	+	-	+	+
E3.13.23	+	+	+	-	+	+	-	+	+
H5.2.3	+	+	-	-	-	-	-	+	+
H5.5.2	+	+	-	+	-	+	-	+	+
H5.6.1	+	-	-	-	-	-	+	+	+

<sup>a</sup>reactivity = +; no reactivity = -

<sup>b</sup>Strains 71, CHA96, CHA0, CHA89, 113, LS107d2, and B52 did not react with any clone tested



the supernatant from *P. fluorescens* ATCC 31732 (code 16) (Table 18). A single clone did not react with all the supernatants tested. However, a combination of clones H5.5.2 and C5.1.18 detected lipase in all strains tested, suggesting a mixture of the Ab from these two clones will detect lipase from many different species. The observed immunological diversity indicates that a variety of lipolytic enzymes are produced in pseudomonads.

**Zymogram analysis.** Supernatants from *P. fragi* 71 (code 5) and *P. fluorescens* RO98 (code 9) were tested using a zymogram to examine the enzyme content of the supernatant. The supernatant from *P. fragi* 71 contained a single band at 41 mm from the origin with lipase activity, and *P. fluorescens* RO98 contained three bands at 15, 37, and 45 mm from origin with activity. This indicated that the two species produced different lipases based on their migration in the urea gel and that *P. fluorescens* RO98 may produce three different proteins with lipase activities. Appearance of multiple activity bands due to aggregation of the lipase can be ruled out since they were treated with a reducing agent ( $\beta$ -mercaptoethanol) and urea prior to electrophoresis (17).

**Isolation of lipase.** Lipase purification by ion-exchange chromatography from the culture supernatant of *P. fragi* 71 grown in McKellar's medium yielded three fractions with lipase activity. These three fractions contained three different proteins with molecular weights of 19.2, 21.5, and 14.4 kDa (Figure 20). The N-terminal sequence of the proteins showed a 100% homology to  $\alpha$ -lactalbumin (14.4 kDa) and  $\beta$ -lactoglobulin (18.4 kDa). However, the third protein (21.5 kDa) contained a unique N-terminal sequence, presumably the same protein detected in the zymogram. These data support the zymogram observation where a single protein band

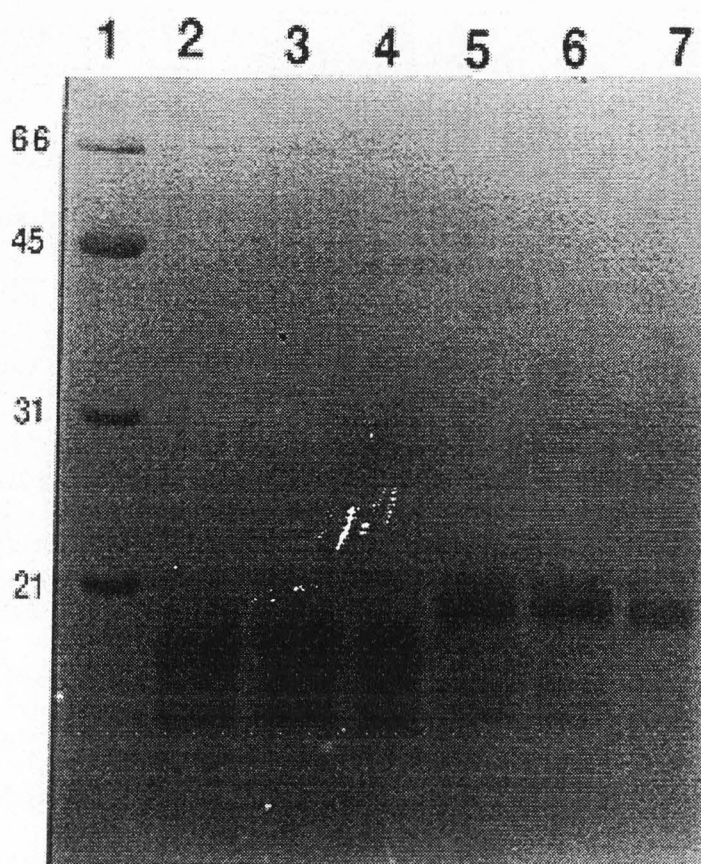


Figure 20. SDS-PAGE of lipase fractions after anion exchange chromatography of *P. fragi* 71 supernatant. Lane 1, molecular weight standards; 66, 45, 31, and 21 kDa, Lane 2-4, fraction 16; Lane 5-7, fraction 19.

was found, and suggest that the lipase associated with the whey proteins during growth. Interestingly, lipase activity was associated and isolated with whey proteins, suggesting that lipase from pseudomonads may associate with these proteins in milk as well. The lipase from *P. fragi* aggregated when free from the whey proteins, as observed on native and SDS-PAGE gels. Further work to purify the lipases from *P. fluorescens* is detailed in Chapter XI.

## DISCUSSION

The aim of this investigation was to determine the influence of medium, strain, and heat stability on lipase activity in pseudomonads related to raw milk and dairy products. This was done by conducting a factorial experiment with various growth media, strain combinations, incubation times, and heat treatments. The data were analyzed with an ANOVA using the sum of duplication and the three-way interactions as the error term. This is a conservative approach to the analysis (pers. comm., Dr. D. Sisson, July, 1998) when replication was not conducted. With this in mind, the conclusions drawn based on this analysis will under-estimate the influence of each factor, compared to using a error term based on replication rather than duplication.

Considerable variation in lipase activity was observed among the treatments tested in this study (Figures 18, 19). Lipase activity was significantly ( $P < 0.001$ ) influenced by medium, strain, incubation time, and pasteurization. Growth medium significantly influenced total lipase activity (Figure 18A). It was observed that lipase was not produced in McKellar's medium in the absence of an inducer consisting of whey proteins, but the activity returned with addition of these proteins, indicating that whey

contains some critical component for lipase activity. This may be linked to the regulation of lipase activity (28, 31), or these proteins may act as chaperones assisting stabilization of the secreted enzyme or may be involved in direct regulation of lipase production. In support of the latter, partial purification of lipase from *P. fragi* 71 showed that the lipase associated with  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin.

None of the media tested contained added lipid, yet all media tested produced lipase activity. Absence of lipids in the growth medium with lipase production was noted by others as well (5, 27). This suggests that induction of lipase has another trigger, perhaps a metal ion as determined for protease production (28) or other proteins (such as whey proteins). While this is an interesting and worthy subject, exploring the regulation mechanism was beyond the scope of this study.

As expected, strains produced significantly ( $P = 0.003$ ) different amounts of lipase (Figure 18B) and significantly ( $P < 0.001$ ) higher lipase activity at 50 h than at 24 h of incubation (Figure 18C and 19B). Increased levels of lipase activity were obtained for all strains except *P. fragi* 71 (code 5) with incubation to 50 h. The increase over time may be due to an accumulation of enzyme activity or production of another enzyme as a secondary metabolite when the cells enter stationary phase, which occurs after about 36 h of growth (4). Rowe and Gilmour (34) and Griffiths (20) also reported an increase in activity during late exponential and early stationary phase of *Pseudomonas* grown in milk. Enzyme production in stationary growth phase may be explained by the need for simple growth nutrients such as peptides, fatty acids, and carbohydrates (35) for survival after these types of nutrients are exhausted during the exponential phase. However, this does not rule out the possibility

that different enzymes may be produced during different stages of growth. Findings suggest that raw milk holding times of > 24 h produce significantly higher levels lipase if the initial level of contamination is at least  $10^7$  cfu/ml. The rate of inoculum in this study was  $10^7$  cells/ml, which compares with raw milk psychrotrophic load (8).

Pasteurization significantly ( $P < 0.001$ ) decreased lipase activity, but the enzyme retained more than 50% of the initial activity following the heat treatment (Figure 18D). Resistance of the enzyme to heat denaturation was greater at 24 h of incubation than at 50 h (Figure 19C). The observed decrease in heat resistance during 50 h of incubation may be due to proteolytic degradation of the lipase because increased levels of protease are produced during stationary phase of growth (34, Chapter IV, Chapter V). Although the proportion of heat resistant activity was lower at 50 h, the total heat resistant activity was greater at 50 h than at 24 h. This further emphasizes the need for prompt processing of milk to avoid the problem with lipolytic spoilage in processed dairy products.

Initial evidence indicating that lipase produced among strains was different was provided by serological analysis. Lack of reactivity with monoclonal antibodies to a commercial *P. fluorescens* lipase revealed that the culture supernatant of each strain tested, particularly 71 and RO98, reacted differently. Strain ATCC31732 was serologically similar to the commercial lipase preparation from *P. fluorescens*, but different from the other strains tested, suggesting the lipase from this strain was sold as the commercial preparation and, therefore, used as the initial antigen. Immunological diversity among the supernatants tested suggests that pseudomonads produce

a variety of lipolytic enzymes that can be detected by using a combination of two hybridoma clones in a rapid test.

Zymogram analyses and microsequencing were done to verify the chromatography results obtained during this study. Zymogram of *P. fragi* 71 (code 5) supernatant yielded a single band, indicating that *P. fragi* 71 (code 5) produced a single lipase. A zymogram of *P. fluorescens* RO98 (code 9) produced three bands with lipase activity. Also, *P. fragi* 71 (code 5) and *P. fluorescens* RO98 (Code 9) produced different lipolytic enzymes based on their migration distance in the urea gel. In addition, the zymogram data established that *P. fluorescens* RO98 (Code 9) produced multiple enzymes with lipase activity. Christen and Marshall (7) found at least eight protein bands having lipolytic activity on a non-denaturing acrylamide disc gel of *P. fluorescens* 27 culture supernatant. They concluded that the wide distribution of lipolytic activity resulted from the presence of isozymes, multiple lipases, polymerization, or hydrolysis of the same enzyme. The technique used in the present study, however, eliminates the possibility of lipase aggregation because sample preparation for electrophoresis involved heating in the presence of  $\beta$ -mercaptoethanol (19).

Anion exchange chromatography of supernatant from *P. fragi* 71 produced three fractions with lipase activity, suggesting three proteins with lipase activity may be produced in this strain. Electrophoresis of the active fractions from *P. fragi* 71 (code 5) demonstrated that each fraction contained a different protein with lipase activity. However, microsequence information of these bands revealed that only one fraction contained a unique sequence with a large amount of another substance, presumably lipopolysaccharide. The other fractions contained whey proteins contaminated with lipase. These



findings indicate that a single lipase was produced in *P. fragi* 71 (code 5). Multiple lipase peaks were previously purified (16, 18, 21, 27). Two lipase peaks were purified from *P. fluorescens* AFT29 by ion-exchange chromatography on DEAE 32 cellulose, but these two peaks were not investigated further (16). Gel filtration of *P. fluorescens* AFT36 culture supernatant yielded three peaks with lipase activity, which was attributed to enzyme aggregation (18). Two lipase peaks with native molecular weights ranging from 25,000 to 250,000 were purified from a *P. fragi* cell-free concentrate (27). The molecular weight differences in this study suggest the two peaks are aggregates (250,000 MW) and individual enzyme units (25,000 MW). Similar results were obtained for *Pseudomonas* sp. KWI-56 (21).

However, none of these studies demonstrated the difference between multiple enzymes and aggregation states to account for the chromatographic results. One explanation for these diverse results may be found by examining the protein structure and chemical characteristics. Jaenicke (22) notes that proteins use at least five mechanisms to stabilize their structure and conformation. If data are viewed in light of the need for stabilization, then it is plausible to propose that lipase will be found in different isolation fractions because it needs other proteins to stabilize it for activity. For example, in this study lipase co-purified with the whey protein inducer in McKeller's medium and with a non-protein component, suspected to be carbohydrate. It was also found to readily aggregate when other proteins were absent. Johnson et al. (23) also noted this during the isolation of lipase from *P. fluorescens* LS107d2. Zymogram, serology, and N-terminal sequence data indicate the diversity of lipases among and within strains tested. These findings taken together indicate that lipase produced by pseudomonads is

diverse and production is influenced by many factors, which may be linked to multiple lipase production in some strains and aggregates in others.

### CONCLUSIONS

Lipase activity varied significantly with strain, growth medium, and incubation time. Thermostability of lipase activity exceeded 60% of the total activity; however, many factors influenced lipolytic activity. Zymogram analyses demonstrated that enzyme production was different between *P. fragi* 71 and *P. fluorescens* RO98, and multiple proteins with lipolytic activity were produced in *P. fluorescens* RO98. The inability of the monoclonal antibodies to react with the lipases from other species and some *P. fluorescens* strains suggests that these bacteria produced different, and in some cases, multiple lipase enzymes, as observed in *P. fluorescens* RO98. Partial purification of the lipase from *P. fragi* 71 supported this observation, despite finding activity in three different fractions and production of a single enzyme that existed in different states. Taken together, these data indicated that the lipolytic enzymes produced by pseudomonads were diverse, and more than one enzyme is produced in a single strain.

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CHAPTER IX  
ISOLATION AND CHARACTERIZATION OF A LIPASE AND AN  
ESTERASE FROM *PSEUDOMONAS FLUORESCENS* RO98

ABSTRACT

Two heat-stable extracellular lipolytic enzymes were purified from the cell-free supernatant of *Pseudomonas fluorescens* RO98 by anion exchange, size-exclusion chromatography, and preparative electrophoresis. The lipolytic enzymes had molecular weights of 50 (Pf-lip1) and 12 kDa (Pf-lip2) as determined by SDS-PAGE. They retained 69 and 53% activity following pasteurization treatment (62.5°C/30 min) with  $D_{62.5}$ -values of 12.7 h and 29.9 h, respectively. The temperature optima were 45 and 35°C for Pf-lip1 and Pf-lip2, respectively. Both enzymes had a pH optimum of 9.0. Pf-lip1 preferred longer chain length fatty acids, with myristate being hydrolyzed fastest. Pf-lip2 preferred shorter chain-length fatty acids, with propionate being hydrolyzed fastest. Gas chromatographic analysis of milk fat hydrolysis revealed that Pf-lip1 hydrolyzed milk fat, whereas, Pf-lip2 did not, indicating that it was an esterase. Pf-lip1 preferred to hydrolyze the Sn-1 and-3 positions of triolein, but Pf-lip2 was inactive against this substrate. This study demonstrates that this strain and most likely other pseudomonads produce multiple lipolytic enzymes simultaneously that impact the storage of thermally processed dairy products.

INTRODUCTION

Lipases (E.C.3.1.1.3) and esterases (EC 3.1.1.1) are hydrolases that act on the carboxyl ester bonds of acylglycerols to liberate free fatty acids and glycerol.



Historically in the dairy industry, lipases are defined as "long-chain fatty acid ester hydrolases" because long chain fatty acids are preferred substrates (3). However, this definition was recently modified to distinguish esterase and lipase activity based on the ability of a lipid-water interface to activate the enzyme. The surface area of the lipid-water interface determines the rate of lipolysis by a lipase, but not an esterase. Due to this requirement, lipases are defined as carboxyesterases acting on emulsified substrates (15). Esterases act on smaller, less hydrophobic lipid substrates and, therefore, do not require an interface for action (25).

Contamination of milk with lipolytic enzymes produced by psychrotrophic bacteria before processing is a concern in long shelf-life dairy products. The exact number of enzymes produced by pseudomonads is not known, nor are the environmental parameters that govern their expression. Production of multiple lipolytic enzymes by several strains of *P. fluorescens* has been indicated by several studies but conclusive evidence is lacking.

Optimum activity characteristics of lipolytic enzymes from the same species are diverse (11). Preparations containing lipolytic activity have an optimum pH range of 7 to 9, an optimum temperature range of 30° to 70°C, molecular weights ranging from 33,000 to 49,905, and highly variable heat inactivation values (9, 11, 17). *Pseudomonas fluorescens* AFT 36 produces three peaks with lipase activity, which is dominated (71% of the total activity) by a single type of lipase (11). This enzyme is stabilized by  $\text{Ca}^{2+}$  and has other characteristics similar to lipases isolated by Bucky et al. (4) and Dring and Fox (9). The other two fractions containing activity were not investigated further, and it was concluded that multiple aggregation forms of the same enzyme were observed. *Pseudomonas fluorescens* 27 produced two to eight different

enzymes with varying fatty acid release profiles on butter oil (34). Further purification and characterization of these enzymes was not done. More than one lipase enzyme seems to be produced by a species and is dependent on growth medium and environmental growth conditions. This, however, is not demonstrated. The objective of this study was to isolate multiple lipases from *P. fluorescens* RO98, thereby demonstrating and confirming this ability in dairy-related pseudomonads.

## MATERIALS AND METHODS

### Strains and Medium

*Pseudomonas fluorescens* RO98, a raw milk isolate, was used for enzyme isolation (13). Working cultures were prepared from frozen culture stocks stored at  $-70^{\circ}\text{C}$  in reconstituted NFDM containing 20% glycerol. Cells were thawed, inoculated into nutrient broth (Difco Laboratories, Detroit, MI), and incubated for 24 h at  $30^{\circ}\text{C}$  with aeration (shaking at 200 rpm). An inoculum for enzyme production was freshly prepared by harvesting the cells by centrifugation ( $6000 \times g$  for 10 min at  $4^{\circ}\text{C}$ ). The cells were washed three times with sterile saline (0.85%). Cells were resuspended to an optical density of 0.2 at 620 nm in sterile saline. This was inoculated (1%) into a vessel containing 14 L of sterile McKellar's minimal salts medium containing 1% inducer (28).

Lipase inducer for McKellar's medium was prepared from UHT skim milk by acidification with 1 N HCl to pH 4.6 to precipitate the caseins followed by centrifugation ( $7000 \times g$  for 10 min at  $4^{\circ}\text{C}$ ) and filtration through a Whatman #1 filter paper (Whatman International Ltd., Maidstone, UK). The filtered whey was neutralized to pH 7.0 using 1 N HCl, filter-sterilized

through a 0.22- $\mu$ m syringe filter (Gelman Sciences Inc., Ann Arbor, MI), and stored at  $-70^{\circ}\text{C}$  until required. This was added (1% v/v) to sterile McKellar's minimal medium. The vessel was incubated at  $25^{\circ}\text{C}$  for 48 h with aeration by constantly purging air through a 0.2- $\mu$ m filter (Amicon Inc., Beverly, MA).

### **Determination of Protein Concentration**

Protein concentration was monitored continuously by measuring absorbance at 280 nm during chromatography (System Gold, Beckman Instruments, Fullerton, CA). For other experiments involving protein concentration measurements, a BCA protein assay kit was used according to the manufacturer's instructions (Pierce Chemical Company, Rockford, IL). Bovine serum albumin was used to construct a standard curve. All assays were conducted in duplicate, and results were expressed as averages.

### **Lipase Assay**

Lipolytic activity was assayed fluorimetrically by measuring the hydrolysis of umbelliferyl myristate (30) (Custom Synthesis; Molecular Probes, Eugene, OR). The enzyme sample was incubated with 1 ml of 50 mM sodium phosphate (pH 7.4) containing 0.2 mM Triton X 100 and 33 nM umbelliferyl myristate. Hydrolysis of the substrate was followed by measuring the increase in fluorescence at excitation and emission wavelengths of 353 and 420 nm, respectively, in a fluorometer after incubation at  $30^{\circ}\text{C}$  for 1 h (Shimadzu corporation, Columbia, MD). Adjustments for the non-enzymatic breakdown of the substrate were made by maintaining negative controls that contained all the components of the reaction mixture except the enzyme. All assays were done in duplicate, and the results are expressed as the means.

## Enzyme Purification

*Preparation of cell free concentrate.* Cells were removed from the culture medium by centrifugation ( $6000 \times g$  for 10 min at  $4^{\circ}\text{C}$ ). The supernatant was concentrated to a final volume of 500 ml using a tangential flow ultrafiltration unit with a 10-kDa membrane (Prep/scale TFF ultrafiltration system; Millipore Corporation, Bedford, MA). This was further concentrated (50-fold) in a stirred-cell ultrafiltration unit with a 10-kDa membrane (YM10 membrane; Amicon, Inc., Beverly, MA) to a final volume of 10 ml. The retentate was filter-sterilized using a  $0.22\text{-}\mu\text{m}$  sterile syringe filter (Amicon, Inc.) and stored in a sterile container at  $-70^{\circ}\text{C}$  until further use. The 10-kDa permeate was also collected, tested for lipase activity, concentrated further using a 1-kDa membrane (YM1 membrane; Amicon, Inc.) in a stirred cell ultrafiltration unit, and stored at  $-70^{\circ}\text{C}$  until further use.

*Purification of Pf-lip1.* The filter-sterilized retentate (500  $\mu\text{l}$ ) was applied to an anion exchange column (Mono Q column HR5/5; Pharmacia Biotech, Uppsala, Sweden) and eluted with a linear salt-gradient of 1 M NaCl in 0.02 M sodium phosphate buffer (sodium phosphate; pH 6.5) at a linear flow rate of 76.5 cm/h. Peaks were collected and assayed for lipase activity. Those peaks with activity were desalted by washing five times using 0.02 M sodium phosphate (pH 6.2) and concentrated in a centrifugal concentrator (Ultrafree 15; Millipore) with a 10-kDa membrane.

The enzyme was further purified by size exclusion on a Superose-12 column (Pharmacia Biotech) equilibrated with 0.02 M sodium phosphate (pH 6.5) and eluted using the same buffer at a linear flow rate of 38.2 cm/h. Peaks were collected and assayed for lipase activity. The peak with lipase activity was concentrated and further purified by preparative electrophoresis.

Partially purified lipase was mixed with an equal amount of double strength electrophoresis sample buffer to a final concentration of 87.5 M Tris-HCl (pH 6.8), 5 mM  $\beta$ -mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue (19). Samples were placed at 32°C for 30 min prior to electrophoresis. A 10% resolving gel (6 cm) and 4% stacking gel (1 cm) were cast in a 28-cm diameter tube (Prep cell Model 491, Biorad, Hercules, CA). The upper and lower tank buffers contained 25 mM Tris-HCl (pH, 192 mM glycine, and 0.1% SDS. Elution buffer was the same as tank buffer with the exception of SDS. The sample was electrophoresed for 6 h at a constant power of 12 W at 4°C after loading sample (2 ml) on the stacking gel. Proteins were eluted continuously at a flow rate of 0.2 ml/min. Fractions were collected at 10-min intervals following the elution of the dye front. The fractions were assayed for lipase activity, and those with activity were pooled and dialyzed against distilled water for 16 h at 4°C and lyophilized.

**Purification of *Pf-lip2*.** The 10-kDa filtrate obtained from the culture supernatant was concentrated using a 1-kDa membrane (YM1 membrane; Amicon, Inc.) in a stirred cell ultrafiltration unit (Amicon, Inc.). This was applied to an anion exchange column (Mono Q; Pharmacia Biotechnology) and eluted as previously outlined. All peaks were collected, assayed for activity, and examined by SDS-PAGE.

### Molecular Weight Determination

Molecular weight of the purified enzymes was estimated by SDS-PAGE and size-exclusion chromatography. Electrophoresis was done with a Phast system (Pharmacia Biotechnology) in reducing conditions using a 8–25% gradient polyacrylamide Phast gel (Pharmacia Biotechnology). Samples were

prepared in Tris-HCl buffer (pH 6.8) containing 2.5% SDS and 1%  $\beta$ -mercaptoethanol. A 10-kDa protein ladder (Life Technologies, Gaithersburg, MD) was used to estimate molecular weight. Proteins were visualized by silver staining the gel following electrophoresis as described by the kit instructions (Plusone; Pharmacia Biotechnology).

The native molecular weight was determined by size-exclusion chromatography on a Superose-12 size-exclusion column (10/30; Pharmacia Biotechnology) and eluted with 20 mM sodium phosphate (pH 6.5) with a linear flow rate of 38.2 cm/h. The column was calibrated with ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa),  $\beta$ -lactoglobulin (36 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa) (Pharmacia Biotechnology).

### **Influence of Temperature and pH**

The optimum pH for the activities of Pf-lip1 and Pf-lip2 was determined by assaying each enzyme at 30°C using 10 mM umbelliferyl myristate as the substrate. The following buffers between pH 3.6 and pH 10.6 were used: 50 mM acetate buffer (pH 3.6 to 5.9), 50 mM MES (pH 4.0 to 7.3), 50 mM Tris-HCl (7.2 to 8.9), and 50 mM carbonate (8.5 to 10.6).

The influence of temperature on lipase activity was determined by assaying lipase activity at temperatures ranging from 5 to 70°C in 50 mM sodium phosphate buffer (pH 7.4) containing 0.2 mM TritonX-100. Buffers were pre-incubated at the respective temperature prior to the assay.

### **Chain-Length Specificity**

Chain-length specificity of the enzymes was determined by the method of Blake et al. (1) using *p*-nitrophenol fatty acids. Activity was calculated as



an increase in  $b^*$  readings (yellow color) and expressed as  $\Delta b^*/h$ . The enzyme (0.25  $\mu\text{g}$ ) was incubated with *p*-nitrophenyl fatty acid conjugates of chain-lengths  $C_4$  to  $C_{18}$ . The assay mixture consisted of enzyme, 50 mM sodium phosphate (pH 7.2), 5 mM substrate in dimethyl sulfoxide (DMSO), and 0.2 mM TritonX-100. Hydrolysis of the substrate was measured by recording  $b^*$  color values continuously for 4 h in a reflectance colorimeter (Omnispec Biomonitor 4000, Wescor, Inc., Logan, UT). Negative controls containing each fatty acid substrate without the enzyme were included. This activity was used to adjust for the spontaneous breakdown of the respective substrate. All reactions were done in duplicate, and the results were expressed as the mean.

#### **Determination of Free Fatty Acids Produced in Milk**

The enzymes (0.8  $\mu\text{g}$ ) were added to 2 ml UHT whole milk (Gossner Foods, Logan, UT) and incubated for 24 h at 4 and 37°C. Release of free fatty acids (FFA) was determined with GC using by the method of de Jong and Badings (6) with the following modifications. Milk samples (2 ml) were extracted in duplicate with 2 ml of GC/HPLC grade ethanol (Burdick and Jackson, Muskegon, MI) and 0.2 ml  $\text{H}_2\text{SO}_4$ . An internal standard solution consisting of  $C_3$ ,  $C_5$ ,  $C_7$ ,  $C_{11}$ , and  $C_{15}$  fatty acids was added to the milk prior to extraction (0.2 ml). Three 2.5-ml volumes of ether-heptane (high purity grade, Burdick and Jackson; 1:1 v/v) were used to extract the sample. The solvent layer was transferred to a flask containing 0.2 g of anhydrous  $\text{Na}_2\text{SO}_4$ . Neutral lipids were removed from the samples using aminopropyl columns (J.T. Baker, Phillipsburg, NJ). Free fatty acids were eluted from the column with diethyl ether containing 2% formic acid and analyzed on a 30-m  $\times$  0.25-

mm capillary column (J and W Scientific Inc, Folsom, CA) using a Shimadzu GC-17A gas chromatograph (Shimadzu, Columbia, MD). Run conditions used to separate the free fatty acids were described by DeJong and Badings (6). A negative control of UHT whole milk was used to account for background levels of FFA. Data are expressed as the mean of replicate experiments and duplicate analyses.

### **Sn-Specificity of Lipases**

Sn-specificity of the two enzymes was determined by gas chromatography of the products formed after the hydrolysis of a triolein emulsion prepared by the method of Talon et al. (36). An emulsion of triolein was prepared using arabic gum, NaCl, and sodium deoxycholate (Sigma Chemicals) in 50 mM Tris-HCl buffer (pH 7.2). A uniform emulsion was obtained by homogenizing the mixture for 5 min in an Ultra-Turra T25 (Janke and Kunkel IKA Labortechnik, Staufen, Germany). The reaction was started with the addition of 0.8  $\mu$ g of each enzyme to the emulsion and incubated at their respective optimum temperatures. Samples were drawn at 0, 30, and 90 min. The FFA were extracted from them as described by Lykidis et al. (23). Samples were incubated with 5 ml of a mixture containing isopropanol:heptane:H<sub>2</sub>SO<sub>4</sub> (40:10:1 v/v/v). The solvents used were of high purity grade (Burdick and Jackson). Products of hydrolysis were extracted with 2 ml heptane (Burdick and Jackson). Following extraction, the solvent was evaporated from the sample by flushing with nitrogen. The mono-, di-, and triacylglycerides were redissolved in pyridine and converted to volatile trimethylsilylether derivatives using N, N-bis (trimethylsilyl) trifluoroacetamide (BSTFA), and trimethylchlorosilane (TMCS) in pyridine

(10). The derivatives were separated by capillary GC (GC-17A; Shimadzu) using a DB XLB capillary column (0.25  $\mu\text{m}$   $\times$  30 m; J & W Scientific) and detected by flame ionization.

### Heat Stability

*D-value determination.* The D-value was determined by heating the enzyme at 62.5°C. The enzymes were diluted 1:20 to a protein concentration of 16  $\mu\text{g}/\text{ml}$  in Jenness-Koops buffer (16), 200  $\mu\text{l}$  of which was dispensed into thin-walled PCR tubes (MJ Research, Inc., Watertown, MA). Samples were heat treated in a thermocycler for different lengths of time from 0 to 24 h (MJ Research, Inc.). Residual activity was expressed as a percentage of the untreated sample.

*Pasteurization stability.* Resistance of the enzymes to pasteurization treatment was determined by heating the enzyme at 62.5°C for 30 min. The enzyme was diluted 1:20 to a protein concentration of 16  $\mu\text{g}/\text{ml}$  in Jenness-Koops buffer (16), 200  $\mu\text{l}$  of which was dispensed into thin walled PCR tubes (MJ Research, Inc.). Samples were heat treated in a thermocycler for 30 min (MJ Research, Inc.). Residual activity was expressed as a percentage of the untreated sample.

### Electroblotting and Microsequencing

Electrophoresis was conducted in reducing conditions using a 160-mm  $\times$  175-mm  $\times$  0.75-mm discontinuous polyacrylamide gel using 5% stacking (pH 6.8) and 14% resolving gel (pH 8.8). Samples and buffers were prepared according to the method of Laemmli (20). The gel was run in a Protean IIXi electrophoresis cell (Biorad Laboratories) for 3 h at a constant current of 30 mA. Following SDS-PAGE, the proteins were blotted to a polyvinylidene

fluoride membrane (Immobilon-PSQ; Millipore Corp.) overnight in a Trans-blot cell (Biorad Laboratories) at a constant voltage of 14 V. The blot was developed with Coomassie stain; lipase bands were excised and sequenced (University of Texas Health Center, Tyler, TX). The N-terminal sequence obtained was compared with other protein sequences in the Swiss-Prot data base.

## RESULTS

### Purification of Enzymes

Anion exchange chromatography of the 10-kDa retentate on a mono Q column yielded two peaks with lipolytic that eluted between 0.2 and 0.4 M NaCl (Figure 21A). These two peaks were collected separately and further purified by size-exclusion chromatography. A single peak with lipolytic activity was eluted in the void volume of the column for each peak from anion exchange chromatography (Figure 21B). The final purification from other proteins after size exclusion chromatography was achieved by preparative electrophoresis (Figure 21C) with a yield of 3.4% (Table 19). This protein was referred to as Pf-lip1.

The 10-kDa filtrate, which also contained lipolytic activity, was also used to purify a second enzyme by anion exchange chromatography (Figure 22). A yield of 0.3% was obtained (Table 20) and this enzyme was designated Pf-lip2.

### Molecular Weight

Each enzyme was found as a self-aggregate with molecular weights >200,000 kDa based on size exclusion chromatography. However, when the

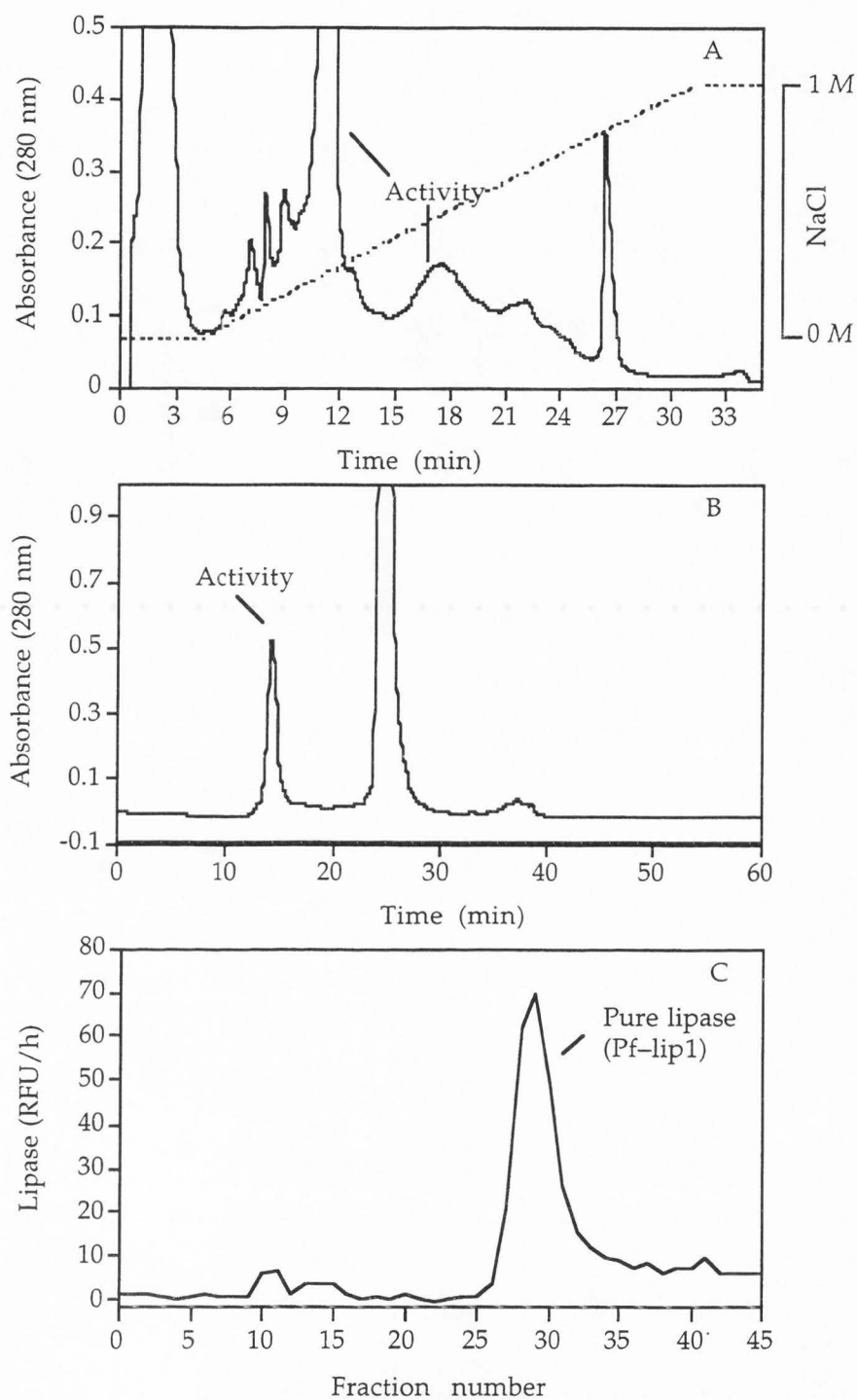


Figure 21. Purification of Pf-lip1 from the 10-kDa retentate of the culture supernatant. Anion-exchange chromatography (Panel A); size-exclusion chromatography (Panel B); and preparative electrophoresis (Panel C).

TABLE 19. Purification scheme of Pf-lip1 from *P. fluorescens* RO98.

Purification step	Total protein (mg)	Total activity (U <sup>a</sup> )	Specific activity (U <sup>a</sup> /mg)	Purification factor	Yield (%)
Crude concentrate	146.4	1144.0	7.8	1.0	100
MonoQ	120.4	1020.0	8.5	1.1	89.2
Superose-12	2.0	871.3	435.6	55.8	76.2
Electrophoresis	0.2	72.7	454.6	58.2	3.4

<sup>a</sup>One unit is defined as 1 RFU/min at 30°C.

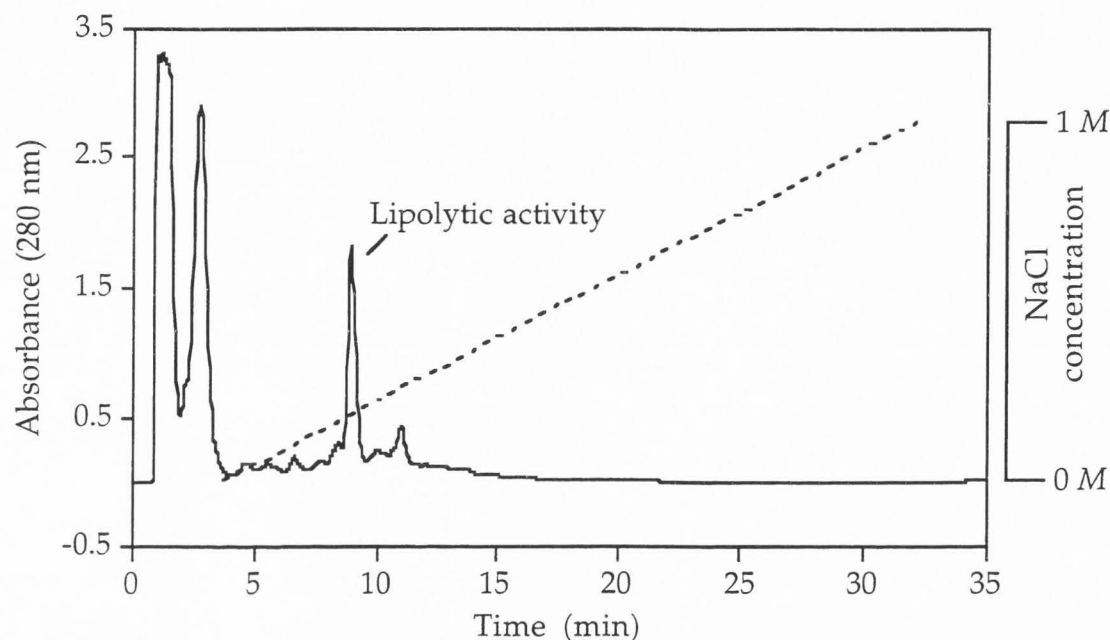


Figure 22. Purification of Pf-lip2 from the 10 kDa filtrate of the culture supernatant. Anion-exchange chromatography of 10 kDa filtrate from *Pseudomonas fluorescens* RO98. Sodium chloride gradient is indicated in dashed lines.



TABLE 20. Purification scheme of Pf-lip2 from *P. fluorescens* RO98.

Purification step	Total protein (mg)	Total activity (U <sup>a</sup> )	Specific activity (U <sup>a</sup> /mg)	Purification factor	Yield (%)
Permeate concentrate	22.1	265.0	0.4	1.0	100.0
MonoQ	0.1	19.4	193.8	472.7	0.3

<sup>a</sup>One unit is defined as 1 RFU /min

two enzymes were resolved using electrophoresis in denaturing conditions, a single protein band was observed for each enzyme. An approximate molecular weight of 50 and 12 kDa for Pf-lip1 (Figure 23A) and Pf-lip2 (Figure 23B), respectively, was observed.

### Temperature and pH Optimum

The optimum temperature for the activity of Pf-lip1 was 45°C (Figure 24A), but no activity was observed at 55°C. Pf-lip2 had a broader temperature range of activity than Pf-lip1 with observed activity between 25 and 55°C (Figure 24B). The optimum temperature was 35°C for Pf-lip2 (Figures 24B). The optimum pH for both Pf-lip1 and Pf-lip2 was 9.0 (Figure 25AB).

### Substrate Specificity

Activity of the two enzymes on fatty acid *p*-nitrophenyl esters of various chain length fatty acids from C<sub>3</sub> to C<sub>18</sub> was determined (Figure 26). Pf-lip1 hydrolyzed all the substrates tested with maximum activity against *p*-nitrophenyl propionate (C<sub>3</sub>). However, Pf-lip2 did not hydrolyze *p*-nitrophenyl myristate (C<sub>14</sub>) and stearate (C<sub>18</sub>), but was most active against caprate (C<sub>6</sub>) and caprylate (C<sub>8</sub>) conjugates.

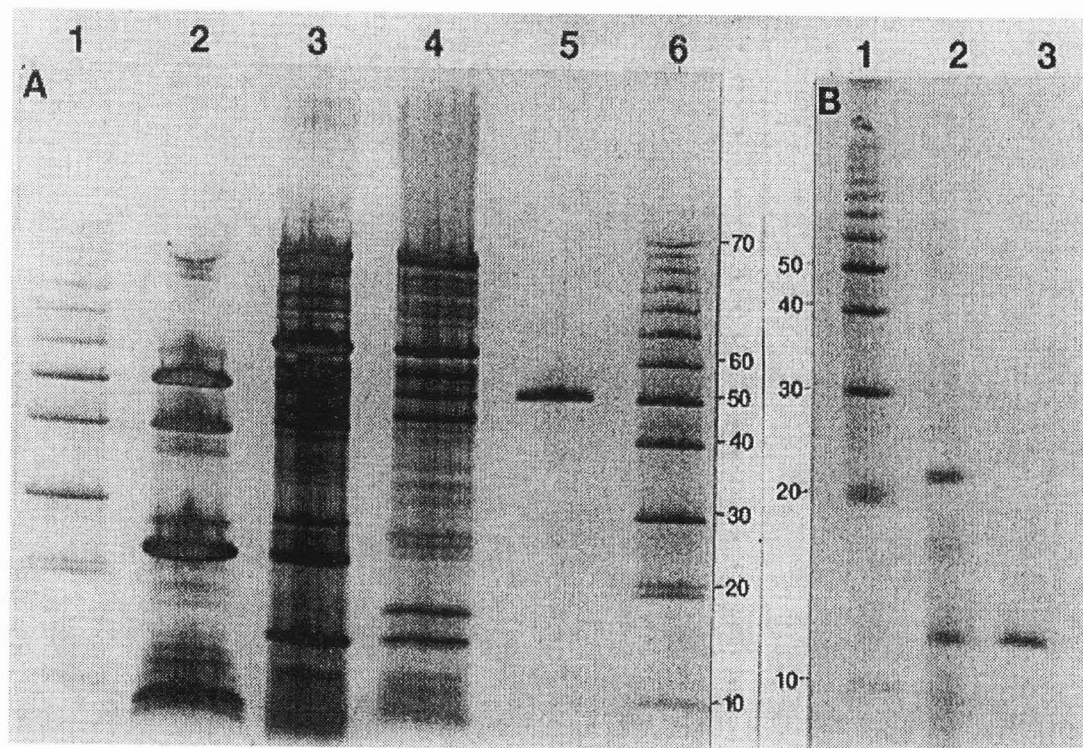


Figure 23. SDS-PAGE analyses of lipase (Panel A) and esterase (Panel B) purification from *P. fluorescens* RO98. Lanes: 1, Crude concentrate (10 kDa retentate); 2, mono Q; 3, size-exclusion; 4, preparative electrophoresis; 5, 10 kDa protein ladder (Panel A); Lanes; 1, Crude concentrate (10 kDa permeate); 2, mono Q; 3, 10 kDa protein ladder.

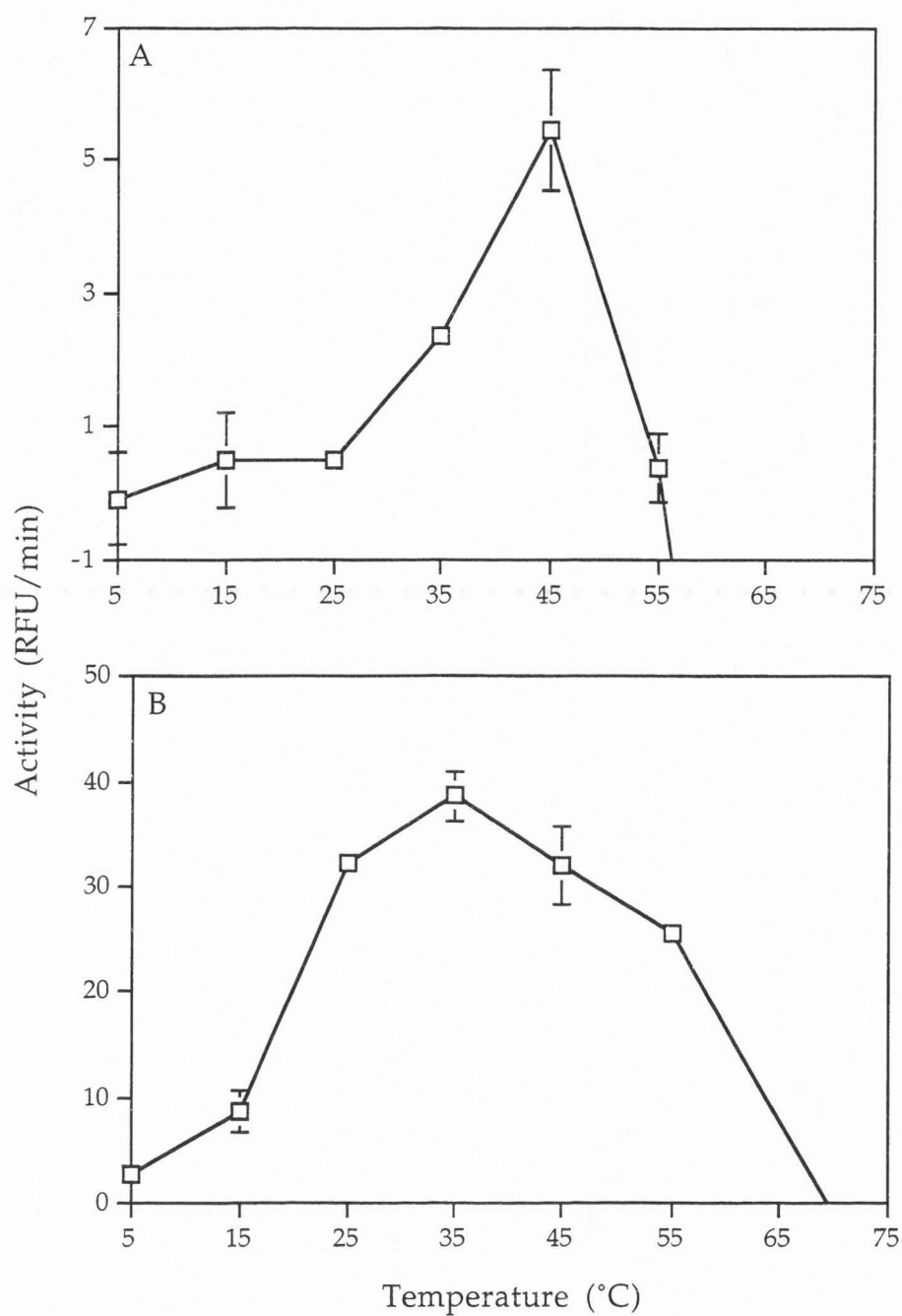


Figure 24. Influence of temperature on activity of Pf-lip1 (Panel A) and Pf-lip2 (Panel B) using umbelliferyl myristate. Error bars indicate the standard deviation of the mean.

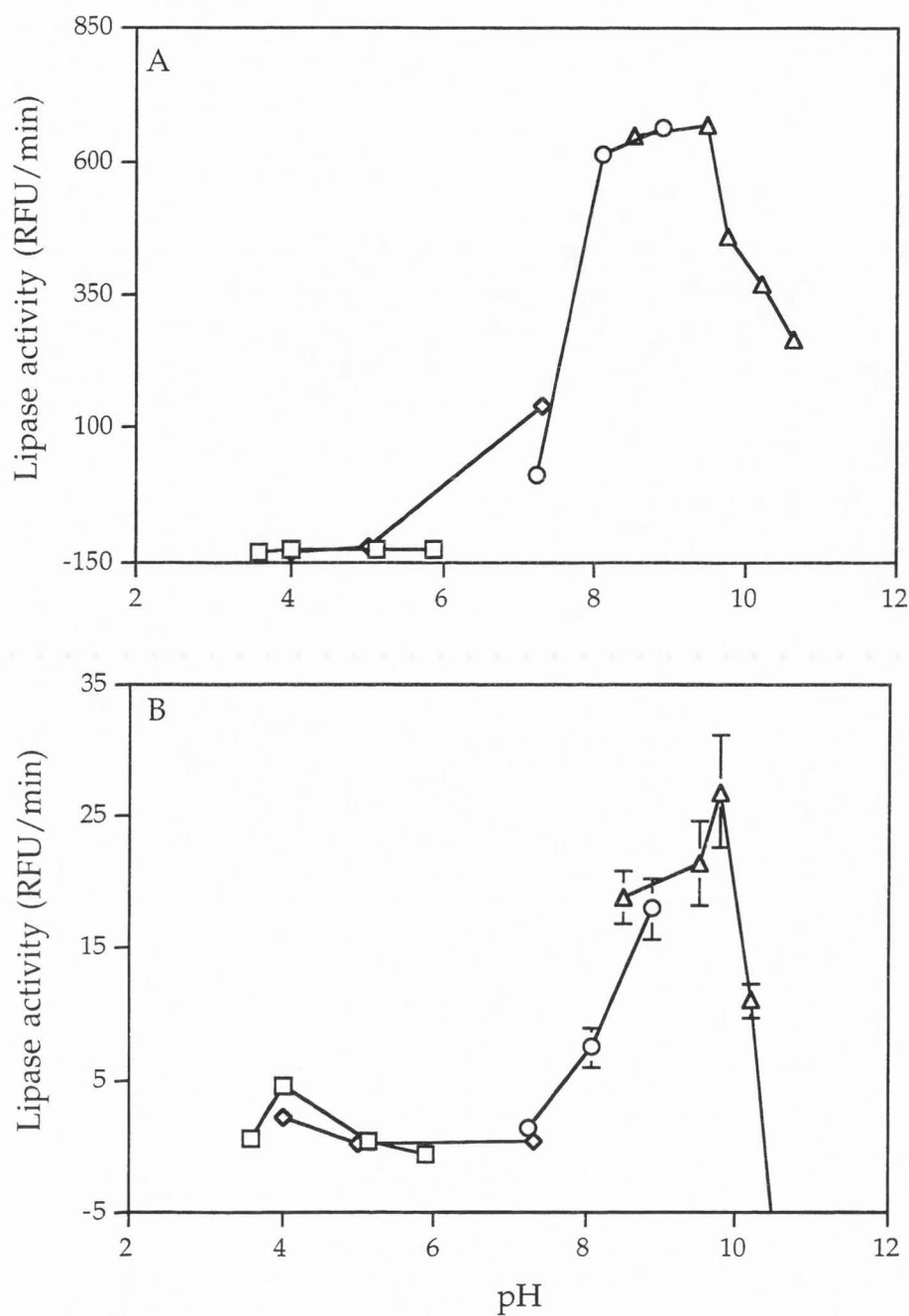


Figure 25. Influence of pH on activity of Pf-lip1 (Panel A) and Pf-lip2 (Panel B), buffers used; acetate (—□—), MES (—◇—), Tris-HCl (—○—), and carbonate (—△—). Results are means of two replications. Error bars indicate the standard deviation of the mean.

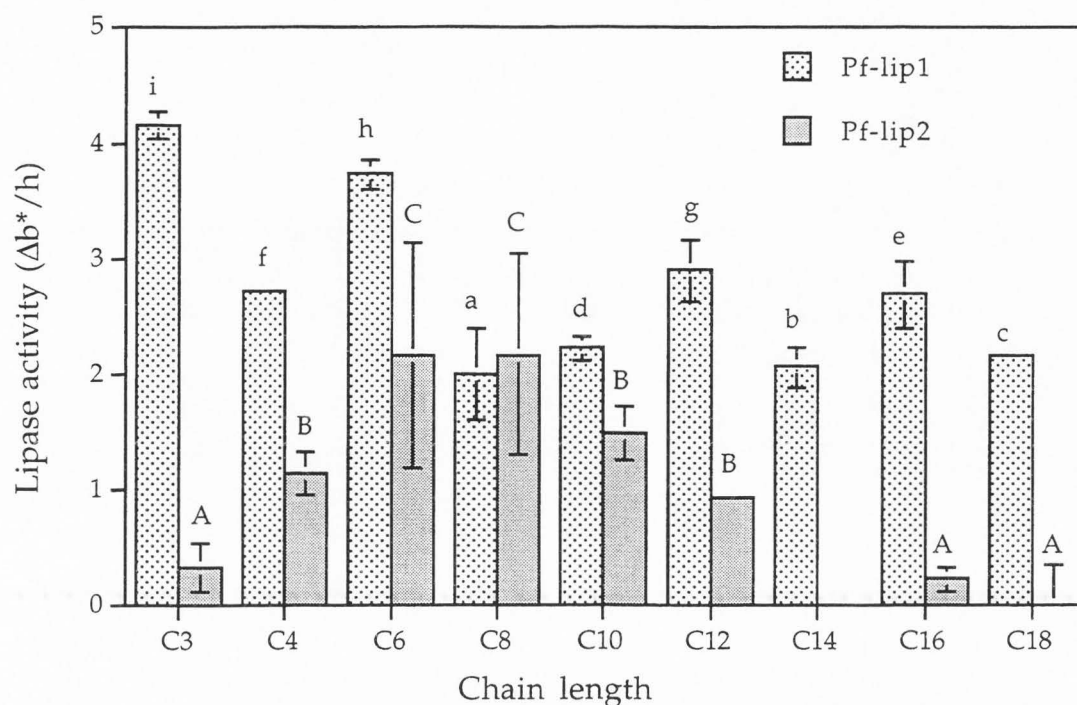


Figure 26. Chain-length specificity of Pf-lip1 and Pf-lip2 with *p*-nitrophenyl esters ( $C_3$  = acetate,  $C_4$  = butyrate,  $C_6$  = caprate,  $C_8$  = caprylate,  $C_{10}$  = caproate,  $C_{12}$  = laurate,  $C_{14}$  = myristate,  $C_{16}$  = palmitate,  $C_{18}$  = stearate,  $C_{18:1}$  = oleate). Error bars represent standard deviation of the means. The letters on top of the bars indicate significant differences between the means when compared using a *t* test ( $\alpha = 0.025$ ). The same letter indicates no significant difference. Comparisons cannot be made between upper and lower case letters.

### Sn-Specificity

The preference of the two enzymes for hydrolyzing a fatty acid in a particular position on the triacylglycerol was determined by incubating each enzyme with triolein. The products of triolein hydrolysis were oleic acid, 1-monoolein, 2-monoolein, 1, 2-diolein, and 1, 3-diolein for Pf-lip1 (Figure 27). Triolein was exhausted by 90 min of incubation and 1, 3-diolein decreased. In contrast, 1-monoolein, 2-monoolein, 1, 2-diolein increased during the same time period, indicating that triolein and 1, 3-diolein were

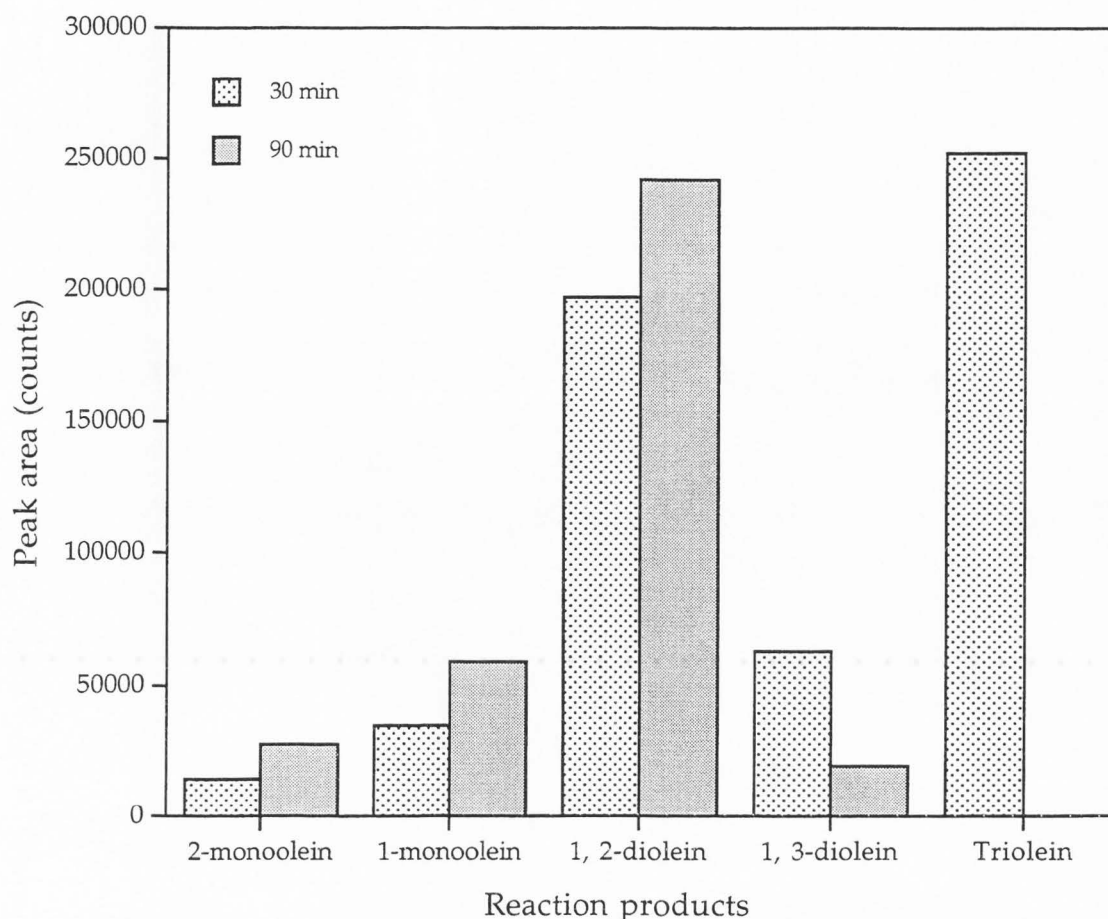


Figure 27. Sn-specificity of Pf-lip1.

the preferred substrates. Pf-lip1 preferred Sn 1 and 3 positions of triolein, but other products were also generated. Pf-lip2 did not degrade triolein, suggesting that it is an esterase and not a lipase.

#### Fatty Acid Release Profiles from Milkfat

Fatty acid release from milk fat was determined by GC analysis. Pf-lip2 was unable to release free fatty acids from milk, further suggesting that it is an esterase. Pf-lip1 released fatty acids with chain lengths from butyric acid ( $C_4$ ) to Oleic acid ( $C_{18:1}$ ). The predominant FFA released at both incubation temperatures Oleic acid ( $C_{18:1}$ ) (Figure 28). The accumulation of butyric acid



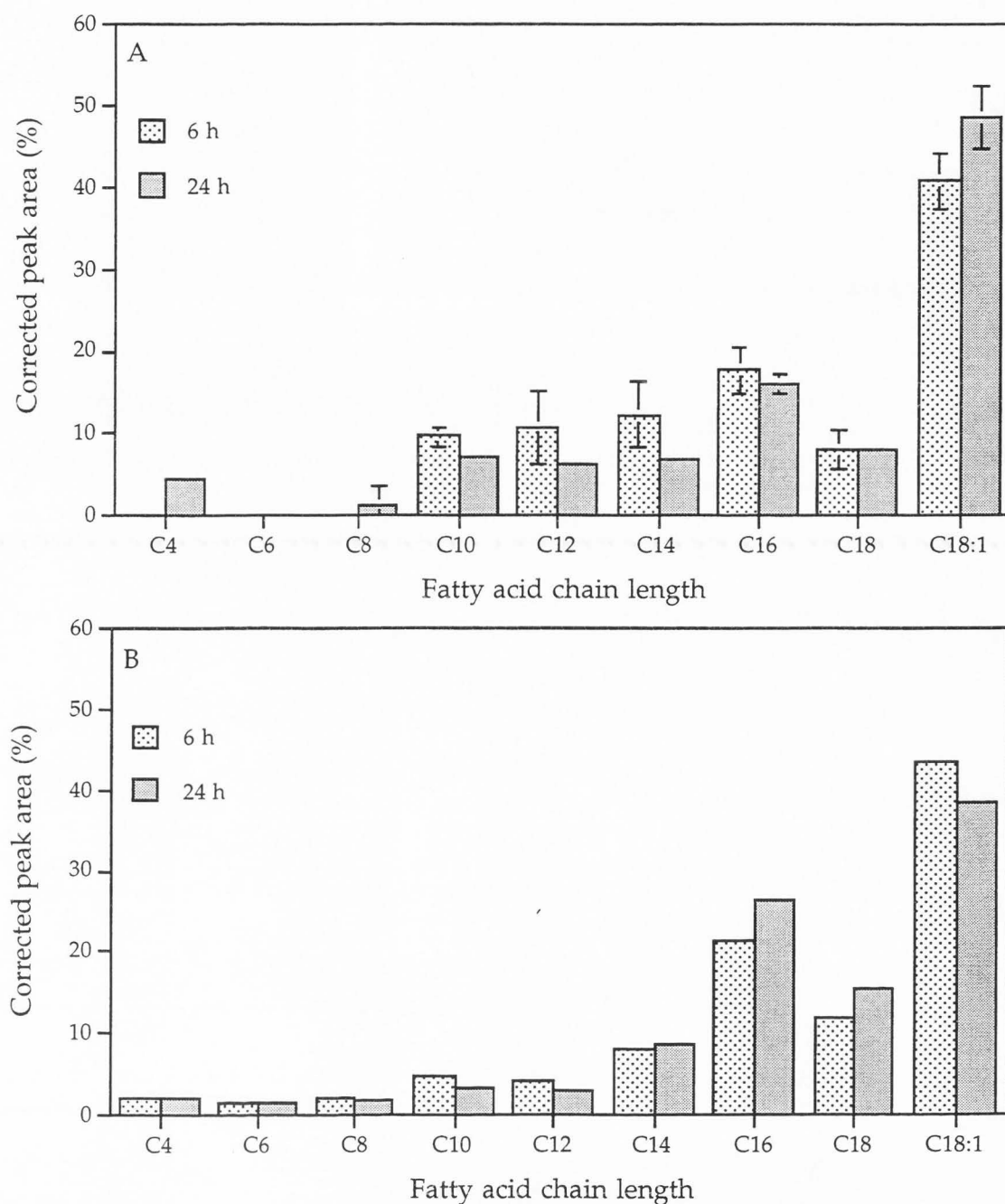


Figure 28. Free fatty acid profiles of milk fat hydrolysis of Pf-lip1 at 4°C (Panel A) and at 37°C (Panel B). Error bars represent standard deviation of the mean. Error bars do not show on panel B due to the small magnitude of error.

(C<sub>4</sub>) doubled at 4°C (Figure 28A) compared to incubation at 37°C (Figure 28B) after 24 h of incubation.

### Heat Stability

Both enzymes retained >50% activity after pasteurization. Pf-lip1 retained a greater proportion (69.9%) compared with Pf-lip2 (53%). The decimal reduction time of the two enzymes was calculated from the denaturation curve obtained by incubating the enzymes at  $62.8 \pm 0.5^\circ\text{C}$  from 0 to 24 h. A rapid loss in activity was observed during the first 4 h of incubation (Figure 29 Inset). The estimated D-values for Pf-lip1 and Pf-lip2 were 12.3 and 29.9 h, respectively (Figure 29).

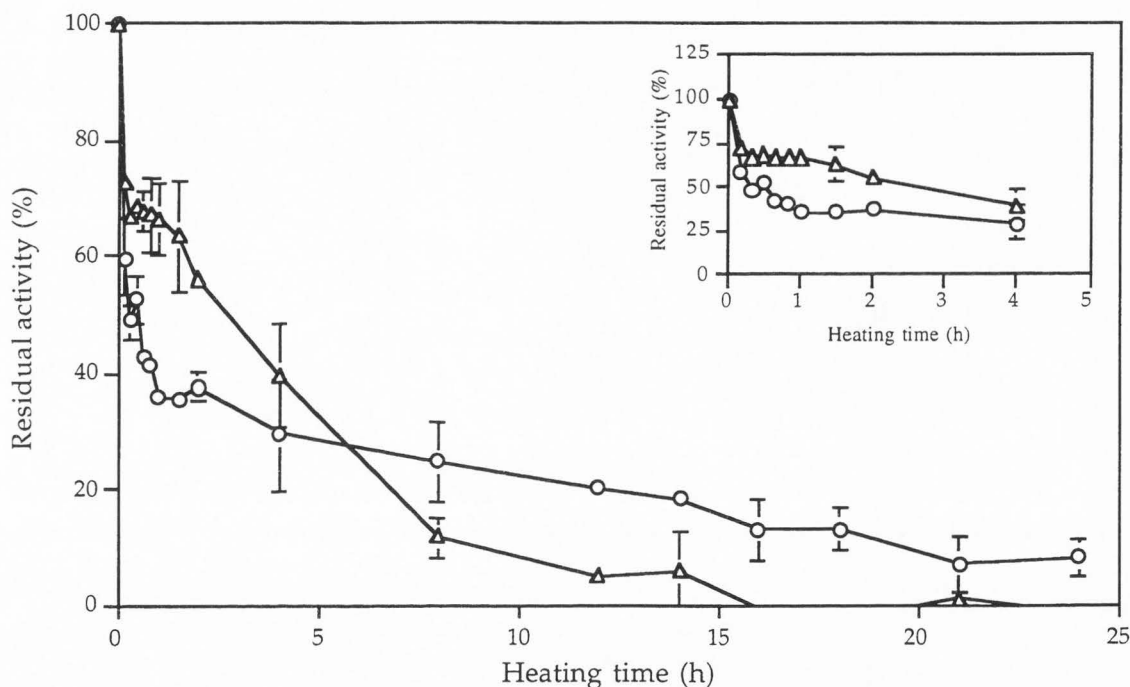


Figure 29. Activity lost during pasteurization over time. Destruction curves of Pf-lip1 (—Δ—) and Pf-lip2 (—O—) at  $62.8^\circ\text{C}$ . Error bars represent standard deviation of the means. The inset graph expands the scale to more clearly show the loss of activity during the first 4 h of heating.

### N-Terminal Sequencing

The N-terminal sequence of Pf-lip1 was determined to be: His-Leu-Ile-Phe-Gly-Tyr-Asn-Asn-Gly-Gly-Xxx-Gln-Xxx-Xxx-Gly-Arg-His-Ala-Pro-Glu. The N-terminal sequence of Pf-lip2 was determined to be Ser-Phe-Leu-Gly-Lys-Leu-Pro-Ser-Ile-Xxx-Lys-Pro-Gln-Gly-His-Xxx-Gln-Pro. No matches were obtained when these sequences were compared to other protein sequences in the Swiss-Prot database using BLAST and FASTA searches, despite containing other lipase sequences from pseudomonads and other bacteria.

### DISCUSSION

The present investigation was conducted to purify the lipases observed in *Pseudomonas fluorescens* RO98 (Chapter VIII). Those studies indicated that multiple enzymes were produced in this strain, opposed to *Pseudomonas fragi* 71, which produced a single enzyme (Chapter VIII). To validate this hypothesis, the culture supernatant of *P. fluorescens* RO98 was concentrated and fractionated with ultrafiltration followed by chromatographic techniques to purify two lipolytic enzymes.

Anion exchange chromatography of the retentate from the culture supernatant yielded two peaks with activity, indicating that two enzymes were produced. After size exclusion chromatography, both peaks eluted in the void volume, yielding a single protein peak with lipolytic activity that was designated Pf-lip1. This suggests that Pf-lip1 interacted with other compounds present during purification in a way that altered interaction with the anion exchange matrix thereby eluting in two peaks. The other component may be carbohydrate (lipo-polysaccharide), which stabilizes other

lipases isolated from *P. fluorescens* LS107d2 (26). Multiple peaks during purification led to the erroneous conclusion among previous researchers that multiple lipases are produced. For example, *P. fragi* NRRL B-25 produced two bands of activity on a zymogram, which separated into a light and a heavy component upon resolution using gradient ultracentrifugation. The heavier enzyme was an aggregate of the lighter one (29). Similar reports are available from *P. fragi* NCDO 752 and *Micrococcus freundenreichii* NCDO 1223 (21). Dring and Fox (9) obtained two peaks with lipase activity by anion exchange eluting at 0 and 0.2 M NaCl concentration (95% activity), respectively. The minor lipase peak (5% activity) was presumed to be different from the major lipase, but due to lack of further investigation it cannot be confirmed that two distinctly different enzymes were produced.

The filtrate obtained after ultrafiltration of the culture supernatant was observed to contain lipolytic activity in addition to that found in the retentate. Consequently, this was used to purify a second enzyme with lipolytic activity which was designated as Pf-lip2. Interestingly, this enzyme also eluted with the same NaCl concentration and was excluded by the size-exclusion column as was observed for Pf-lip1. Although similarities were observed during purification, characterization of the two purified enzymes showed interesting differences of practical importance. This is the first report of two different lipolytic enzymes being isolated from the same supernatant from *Pseudomonas*.

The estimated molecular mass of Pf-lip1 and Pf-lip2 as determined by SDS-PAGE was 50 kDa and 12 kDa, respectively. The reported values for molecular weight range from 16 to 250,000 kDa for other psychrotrophic bacterial lipases (2, 8, 12, 19, 24, 31). The large molecular weight is likely a

result of aggregation of the enzyme. Both Pf-lip1 and Pf-lip2 were excluded by the size exclusion column with a pore size of  $2 \times 10^6$  kDa, suggesting that these two enzymes may aggregate. However, Pf-lip2 passed through a 10-kDa ultrafiltration membrane in a solution containing a high, but undefined salt content, suggesting that this enzyme may aggregate in response to ionic forces.

Pf-lip2 had a broader temperature range of activity than Pf-lip1 with activity between 25 and 55°C. Both the enzymes were active in the alkaline pH range. The implications of raw milk contamination with these enzymes depend on the temperature of storage and the products processed from the milk. For example, Pf-lip1 was more active at milk pH (6.8) and higher storage temperatures. This enzyme may be a problem in fluid dairy products such as UHT milk. In contrast, Pf-lip 2 was active over a wide temperature range, at alkaline and acidic pH values, indicating that this enzyme can be a problem in both fluid and fermented dairy products. Cheese ripening conditions (pH 5.2; 5 to 13°C) provide conducive conditions for Pf-lip2 activity, which may lead to rancidity.

Pf-lip1 hydrolyzed all the synthetic substrates tested. Reports of chain length preference of lipases are diverse. A *P. pseudoalcaligenes* F111 lipase favored  $C_{12}$  and  $C_{14}$  substrates (22), while a *P. fluorescens* MC50 lipase was most active against  $C_8$ . Another *Pseudomonas* spp. lipase exhibited maximum activity against  $C_{10}$  (2, 14, 19). Pf-lip2 hydrolyzed all but two synthetic substrates with the most activity on C6 and C8 (Figure 26). Because it is not possible to distinguish between a lipase and an esterase using *p*-nitrophenyl substrates (26), activity tests on milk fat was done for each enzyme.

Pf-lip2 did not release fatty acids when incubated with milk fat, indicating that it was an esterase. Pf-lip1 hydrolyzed milkfat, and a greater proportion of butyric ( $C_4$ ), caproic ( $C_{10}$ ), lauric ( $C_{12}$ ), and myristic ( $C_{14}$ ) acids were released during incubation at  $4^\circ\text{C}$  opposed to  $37^\circ\text{C}$ . This implies that rancid off-flavors may be formed during refrigerated storage in milk containing this enzyme. McKay et al. (27) report that the principal fatty acids that accumulated from *P. fluorescens* LS107d2 grown in milk were  $C_4$ ,  $C_{16}$ ,  $C_{18}$  and  $C_{18:1}$ . On the contrary, short chain fatty acids were released from UHT milk by a partially purified lipase from *P. fluorescens* 27 (34). High levels of free short chain fatty acids ( $C_4$  to  $C_8$ ) give rise to rancid flavors, but fatty acids between chain lengths of  $C_{11}$  to  $C_{12}$  result in soapy, unclean, and bitter flavors (15). Taking this with the pH and temperature optima, it is reasonable to conclude that activity of this enzyme in fluid milk will result in rancid, soapy, and bitter flavors in the finished product. While activity of Pf-lip2 in fluid milk will not contribute flavor changes from action on milk fat, this enzyme may play a role via the degradation of free triglycerides or fatty acid esters.

Pf-lip2 did not hydrolyze triolein, contradicting the earlier hypothesis that this enzyme may act on free triacylglycerols in milk, resulting in flavor defects during milk storage. Consequently, the role of Pf-lip2 in milk and fermented dairy products is unclear. Examination of the exact substrate for this in milk was beyond the scope of this study and is an interesting issue that needs to be resolved. Lipase Pf-lip1 hydrolyzed triolein resulting in four products. 1, 3-diolein accumulated and then decreased during the 90-min incubation, indicating this was produced from triolein and subsequently became a substrate for the enzyme. The products from this reaction would be 1-monoolein, 3-monoolein, glycerol, and oleic acid. One-monoolein



continued to increase during the incubation period, despite depletion of triolein. Unfortunately, 3-monoolein was not measured which would allow exact preference determination. Presumably, this enzyme has a 1, 3 specificity because it is the most common preference among microbial lipases, including *Aspergillus niger*, *Rhizopus delemar*, *Mucor miehei*, and *Pseudomonas* spp. (12, 21, 24, 32, 33).

Both the esterase (Pf-lip2) and the lipase (Pf-lip1) were thermostable to pasteurization. Initial loss of activity was rapid, followed by a slower loss in activity. Biphasic inactivation curves are characteristic features associated with lipolytic enzymes originating from psychrotrophic bacteria (5, 8, 11, 12, 24, 35). Biphasic inactivation curves are observed when two enzymes catalyze the same reaction in partially purified enzyme (7). In homogenous preparations of lipase, the biphasic inactivation phenomenon can be explained by the presence of two different active conformations of the same enzyme. Aggregation of the enzyme to varying degrees owing to increased hydrophobic interactions between protein molecules at higher temperatures can also explain this phenomenon. Psychrotrophic bacterial lipases show an increase in hydrophobicity at pasteurization temperatures (18, 24), which may account for biphasic inactivation curves. Aggregation of the enzymes may offer a possible explanation because both the enzymes isolated in this study were excluded by the size-exclusion column during purification.

Esterases have not been previously purified from pseudomonads. An esterase gene was cloned and sequenced from a psychrotrophic pseudomonad LS107d2 (26). The molecular mass of the esterase was determined to be 42.2 kDa from the gene sequence, opposed to 12 kDa of Pf-lip2. The activity in LS107d2 was associated with the cell-wall, but was found free in the

supernatant. Although it is possible Pf-lip2 is an intracellular, or a cell-wall bound esterase, it is unlikely. The large quantity of enzyme needed for purification with only a 0.3% yield requires that a large amount of cell lysis occurred which may result in the broth clearing due to cell damage. This was not observed during growth or supernatant harvesting protocols.

The existence of multiple lipases with different heat resistances and other properties intensifies the problem of lipolytic spoilage in milk and makes their detection and control difficult. Production of multiple lipolytic enzymes has been suggested previously but characterization of purified enzymes has not been done in order to demonstrate that different lipolytic enzymes are produced.

### CONCLUSIONS

Two enzymes with lipolytic activity were isolated from *P. fluorescens* RO98. Biochemical characterization of the two enzymes revealed that one of the enzymes was an esterase and the other a lipase. Molecular weights were 50 and 12 kDa for the lipase (Pf-lip1) and the esterase (Pf-lip2), respectively. Both the enzymes were thermostable and retained at least 50% of their original activity after pasteurization. Pf-lip1 was most active against all substrates and Pf-lip2, against medium chain length synthetic substrates. Pf-lip2 was unable to hydrolyze milk fat and triolein. The temperature optima were 45 and 35°C for Pf-lip1 and Pf-lip2, respectively. Both had the most activity at pH 9.0. This is the first report of isolation of two different lipolytic enzymes within a single strain. Isolation and biochemical characterization of lipase purified during this investigation demonstrates that one extracellular lipase and one esterase were secreted by *P. fluorescens* RO98. The synergistic

role of these two enzymes needs further investigation to determine their part in flavor-associated changes in milk and dairy products.

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## CHAPTER X

### SUMMARY AND CONCLUSIONS

Heat-stable extracellular enzymes produced by pseudomonads are of concern to the dairy industry because they remain active in processed dairy products. Diversity of biochemical properties of isolated enzymes of partially purified preparations indicate the production of multiple enzymes in *Pseudomonas*, which may be produced in response to different environmental growth conditions. This has not been confirmed using traditional enzymology. Hence, the aim of this study was to investigate extracellular enzymes from pseudomonads.

### HYPOTHESIS

Psychrotrophic pseudomonads produce a single phospholipase, protease, and lipase that are heat-stable and lead to spoilage in dairy products.

### OBJECTIVES

1. Screen 17 strains of *Pseudomonas* for total and heat-stable lipase, protease, and phospholipase activity.
2. Determine if incubation time has an influence on total and heat-stable phospholipase, lipase, and protease activity.
3. Determine if medium has an influence on total and heat-stable phospholipase, protease, and lipase activity.
4. Isolate and characterize lipase (s) from *Pseudomonas fluorescens* RO98.
5. Isolate and characterize protease (s) from *Pseudomonas fluorescens* RO98.



6. Determine ability of protease to hydrolyze hydrophobic peptides found in Cheddar cheese.
7. Investigate an immunomagnetic method for protease assay.

### INFLUENCE OF GROWTH CONDITIONS ON PHOSPHOLIPASE, PROTEASE AND LIPASE PRODUCTION IN *PSEUDOMONAS*

The influence of culture conditions on the production and thermostability of phospholipase, protease, and lipase production was investigated. Seventeen raw milk and environmental isolates were grown aerobically in nutrient broth, McKellar's minimal salts medium, Chrisope's medium, and skim milk at 8°C with aeration. Samples were collected at 24 and 50 h and split into two subsamples, one of which was pasteurized and the other was left untreated. Enzyme activity was assayed in both samples.

#### Phospholipase

Phospholipase activity varied significantly ( $P < 0.001$ ) with medium, strain, incubation time, and heat treatment. PLC activity was observed in all the media tested and in all the strains except *P. fragi* 71 and *Pseudomonas* sp. 345. In all cases, increased incubation time resulted in higher and more heat-stable PLC activity than shorter incubation times. Growth in skim milk resulted in significantly higher PLC activity compared to Chrisope's medium. Hence, it is likely that raw milk will contain PLC activity at different amounts depending on the strains present and the time held before processing based on the observations of this study. Elimination of this enzyme was not accomplished by heat processing alone; however, a reduction was observed

after pasteurization. Milk containing high PLC activity may not be suitable for long shelf-life products, such as milk powder or UHT milk, due to subsequent defects that will appear during storage.

### Protease

Protease activity was significantly influenced by growth medium and incubation time. Skim milk and nutrient broth produced the highest amount of proteolytic activity, suggesting that dairy products may contain protease. Increasing incubation time also resulted in increased protease activity. However, after 50 h of incubation, the enzyme was heat labile, in contrast to the enzyme produced at 24 h, suggesting that different proteases were produced in stationary phase compared to exponential growth phase. Further purification and characterization of the proteolytic enzymes from these strains is required to prove that different enzymes were produced.

### Lipase

Lipase activity varied significantly with strain, growth medium, and incubation time. Skim milk was a good medium for lipase production. Because strains responded to different media by producing varying amounts of both total and heat-stable activity, considerable variations can be expected in industrial milk supply due to seasonal variations. Generally, longer incubation times resulted in increased enzyme activity. Milk held for more than 48 h prior to processing will contain more enzyme activity. Thermostability of lipase activity exceeded 60% of the total activity. Zymogram analysis demonstrated that *P. fragi* 71 produced a single band with lipolytic activity, but *P. fluorescens* RO98 contained multiple bands with lipolytic activity. The inability of monoclonal antibodies to react with the

lipases from other species and some *P. fluorescens* strains suggests that these bacteria produced different enzymes. This observation further supported the theory for multiple lipase production. Partial purification of the lipase from *P. fragi* 71 supported this observation as well, despite finding activity in three different fractions and production of a single enzyme that existed in different states with whey proteins associated. Taken together, these data indicated that the lipolytic enzymes produced by pseudomonads were diverse, and more than one enzyme was produced.

### ISOLATION AND CHARACTERIZATION OF A PROTEASE FROM *PSEUDOMONAS* *FLUORESCENS* RO98

Protease purification was done to investigate the production of multiple proteases. A single protease was isolated from *P. fluorescens* RO98 with a molecular weight of 52 kDa as determined by SDS PAGE and gel-filtration chromatography. The enzyme was determined to be a zinc metalloprotease with pH and temperature optima for activity of 5.0 and 35°C, respectively. It was thermostable with a  $D_{55}$  of 41 min and a  $D_{62.5}$  of 18 h. The enzyme had a preference for  $\alpha$ -casein as indicated by its low  $k_M$  when tested with individual caseins. Artificial casein micelles were used to determine the preference of the enzyme for a particular casein fraction within a micelle and kinetic parameters. The enzyme hydrolyzed  $\kappa$ -casein preferentially when incubated with the artificial casein micelles. Detection and control of protease activity is critical because the enzyme is able to resist pasteurization and remain active at the pH and temperature of fluid and fermented dairy products. The impact in fermented products is unknown. The impact of this

enzyme may be detrimental in fluid milk for long shelf-life dairy products. These data demonstrated that a single extracellular protease was produced in *P. fluorescens* RO98.

### RAPID DETECTION OF EXTRACELLULAR *PSEUDOMONAS FLUORESCENS* PROTEASE

Although many sensitive assays exist for protease, they are not suitable for detection of protease activity in milk due to the competition of milk proteins with the substrate and the turbid nature of dairy products. Reported immunoassays are sensitive to nanogram levels, but take about 6 h to complete. An immunoassay was explored to overcome these limitations. Antibodies raised to the purified *P. fluorescens* RO98 protease cross-reacted with proteases produced by about 50% of the isolates used in the study within 2 h. The protease was active in the presence of the antibodies, indicating that a test can be developed that will determine the ratio of total enzyme to active enzyme after removal from milk. These antibodies were immobilized on 2.8- $\mu$ m diameter paramagnetic-polystyrene and 7-mm ceramic beads, after which they were used to capture the enzyme from milk and buffer followed by detection with a solid phase sandwich ELISA. By using this assay, total protease was detected both in milk and buffer within 2 h. Protease was detected at a level of 427 ng, but levels of protease lower than this need to be tested to improve the sensitivity of the assay. Further work is needed to increase the sensitivity and broaden the number of pseudomonad proteases detected.

## HYDROLYSIS OF BITTER PEPTIDES BY PROTEASE

The ability of the purified protease to hydrolyze hydrophobic peptides associated with bitterness in Cheddar cheese was investigated. The protease hydrolyzed two hydrophobic peptides associated with bitterness in Cheddar cheese. The  $\alpha_{s1}$ -casein f1-9 was reduced by 50% during the 90-min incubation, and the all the  $\beta$ -casein f193-209 peptide was converted to other hydrolytic products. The results indicate that the protease has the potential to debitter Cheddar cheese as it shows the ability to hydrolyze hydrophobic peptides generated by rennet and starter enzymes. The ability of the protease to hydrolyze hydrophobic peptides preferentially in the presence of less hydrophobic peptides needs to be addressed in the future.

## ISOLATION AND CHARACTERIZATION OF A LIPASE AND AN ESTERASE FROM *PSEUDOMONAS FLUORESCENS* RO98

Lipase isolation and characterization were done in *P. fluorescens* RO98 to verify the observations in Chapter VIII. Two lipolytic enzymes were isolated with molecular weights of 50 (Pf-lip1) and 12 kDa (Pf-lip2). The enzymes were thermostable and retained 69 and 53% activity following pasteurization treatment (62.5°C/30 min) with D-values of 12.7 h and 29.9 h, respectively. Pf-lip1 was most active against all substrates and Pf-lip2 against medium chain length synthetic substrates. Pf-lip2 did not hydrolyze milk fat and triolein. The temperature optima were 45 and 35°C for Pf-lip1 and Pf-lip2, respectively. Both had the most activity at pH 9.0. This was the first report of isolation of two different lipolytic enzymes within a single strain. Isolation and biochemical characterization of lipase purified during this

investigation demonstrate that one extracellular lipase and one esterase were secreted by *P. fluorescens* RO98. The synergistic role of these two enzymes needs further investigation to determine their part in flavor-associated changes in milk and dairy products.

## CONCLUSIONS

The original hypothesis stated that pseudomonads produce a single heat-stable phospholipase, protease, and lipase. The hypothesis was inconclusive for PLC, accepted for protease, and rejected for lipase with regard to the number of enzymes produced. Enzyme production changed in response to growth medium, strain, and incubation time in each case. Residual activity following pasteurization varied via interactions with medium, strain, and incubation time. Immunological cross-reactivity of culture supernatants of 17 *Pseudomonas* strains using antibodies raised to a purified protease and lipase indicated diversity of these two enzymes among the strains. Isolation and characterization of protease from *P. fluorescens* RO98 indicated production of a single protease, proving the hypothesis for this strain. Zymograms of culture supernatants suggested production of multiple lipolytic enzymes. Subsequently, two different lipolytic enzymes were isolated from *P. fluorescens* RO98. Taken together, it is reasonable to conclude that pseudomonads produce a battery of heat-stable enzymes that are active in raw milk, which is a good medium for production, and would remain active in processed dairy products. While they may cause spoilage from off-flavor development, they may also provide benefits, such as debittering cheese. Rapid assays to detect these enzymes will lead to



processing strategies that minimize these enzymes in long shelf-life dairy products.

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## APPENDIX

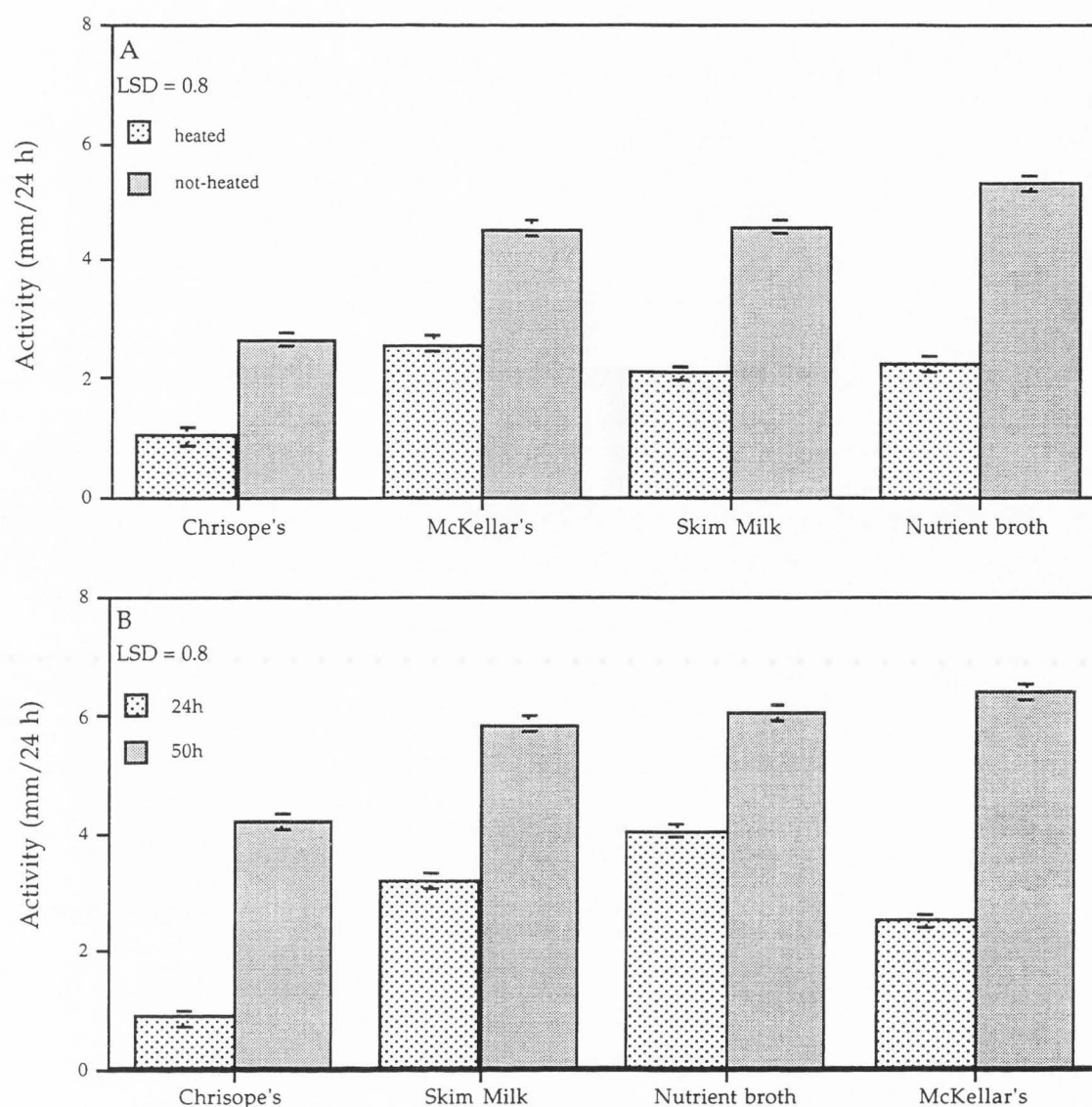


Figure 30. Panel A shows the influence of medium  $\times$  incubation time, panel B shows the influence of medium  $\times$  treatment ( $62.5 \pm 0.5^\circ\text{C}$ ) on phospholipase activity. Activity has been averaged over the other factors. Strain numbers correspond to the strain names in Table 7. Error bars represent standard error of mean.

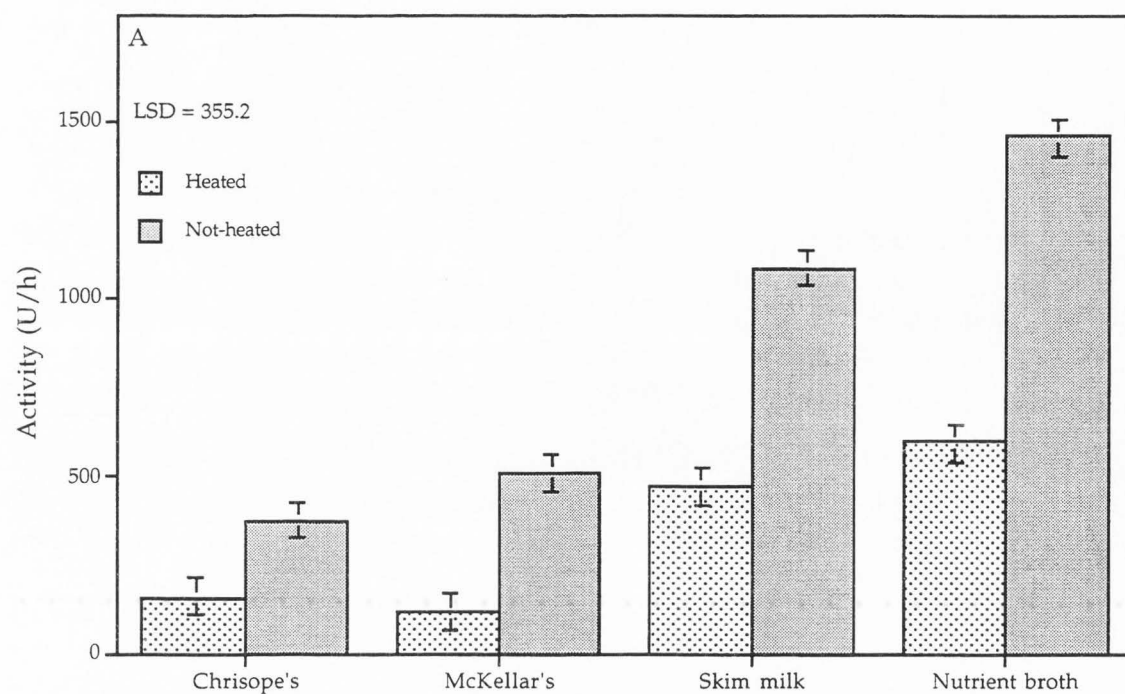


Figure 31. Influence of medium  $\times$  treatment on protease activity. Error bars represent standard error of mean.

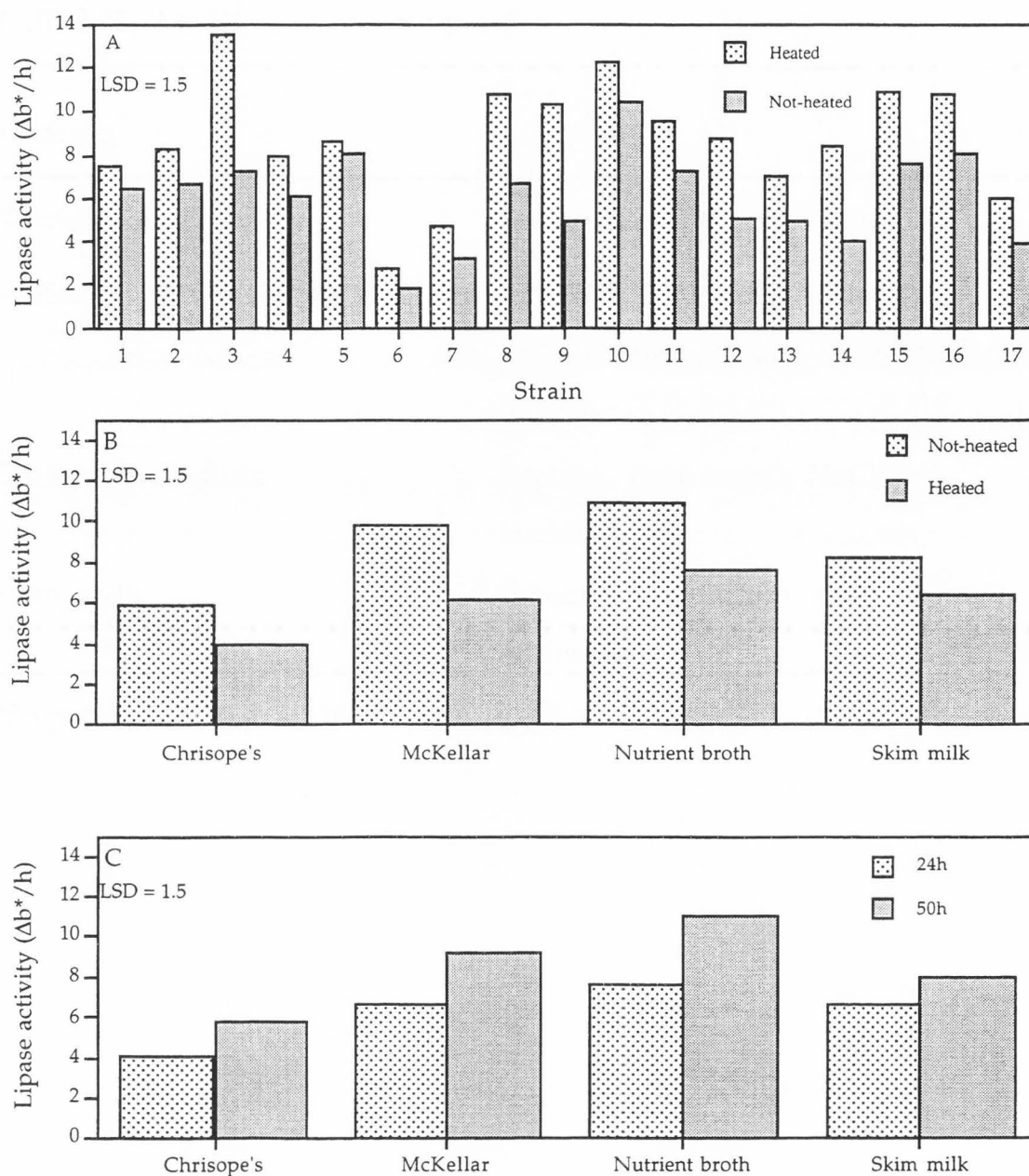


Figure 32. Panel A shows the influence of strain x heat treatment (62.5°C for 30 min), panel B shows the influence of medium x incubation time and panel C shows the influence of medium x heat treatment (62.5°C for 30 min) on lipase activity. Strain numbers correspond to the strain names in Table 16.

TABLE 33. Media used and composition.

Medium	Components
Nutrient broth	Beef extract and peptone
McKellar's minimal salts medium	BES, $\text{NH}_4\text{Cl}$ , $\text{MgSO}_4$ , $\text{NaH}_2\text{PO}_4$ , Sodium Pyruvate, and filtered whey inducer*
Chrisope's medium	Peptone, yeast extract, $\text{NaCl}$ , and $\text{Na}_2\text{HPO}_4$
Skim milk	Casein, whey, lactose, vitamins, and minerals

\*Preparation is detailed in chapters III, IV, and VIII



## CURRICULUM VITAE

Rathna Koka

### EDUCATION:

Ph.D in Food Science, Utah State University, Logan, Utah (expected 1999).

M.S in Food Technology, G. B Pant University of Agriculture and Technology, Nainital, India.

B.S in Agriculture, University of Agricultural Sciences, Bangalore, India.

### WORK EXPERIENCE:

PHD CANDIDATE, Department of Nutrition and Food Sciences, Utah State University, Logan, Utah (1993 to present).

TEACHING ASSISTANT, Department of Nutrition and Food Sciences, Utah State University, Logan, Utah (1994 to '98).

RESEARCH SCIENTIST, Research and Development center, Britannia Industries Ltd., Madras, India (1992-1993).

MASTER'S CANDIDATE, Dept. of Food Technology, G.B. Pant University of Agriculture and Technology, Nainital, India (1989-1991).

RESEARCH TECHNICIAN, Mysore Fruit Products, Bangalore, India (1988).

### AWARDS AND HONORS:

- First place in student research presentation (Intermountain ASM, 1998).
- Outstanding student chapter member award (IFT National Division, 1996-97).
- First place in student research presentation (Intermountain ASM, 1996).
- National Dean's list (1993-1994).
- Honors roll at Utah State University (1993-96)
- Gandhi Graduate Fellowship (Utah State University, 1995-1998).
- Phyllis R Snow graduate scholarship (Utah State university, 1994-1995).
- Presidential fellowship (Utah State University, 1993-1994).
- Ranked first in a nationwide written competitive exam conducted by the Indian Council of Agricultural Research (ICAR) and awarded Junior Research Fellowship (1988).
- Awarded merit scholarship by the department of Biochemistry, University of Agricultural Sciences, India for the year 1988-89.

The award is given to the top scoring candidate in the departmental entrance examination.

#### **PUBLICATIONS:**

1. Studies on the preparation of banana juice by liquefaction with pectinolytic enzymes. 1991. Thesis submitted to the Govind Ballab Pant University of Agriculture and Technology, Nainital, India.
2. A semi-automated reflectance colorimetric method for the determination of lipase activity in milk. 1996. M.R.Blake, R.Koka, and B.C.Weimer. J. Dairy Sci. 79:1164-1171.

#### **PRESENTATIONS:**

1. Isolation and characterization of two lipases from *Pseudomonas fluorescens* RO98. R. Koka and B. Weimer. Presentation at the annual Intermountain Conference of the American Society of Microbiologists (1998).
2. Investigation of thermostable lipases from raw milk isolates of *Pseudomonas* . R. Koka, M. Hill, M. Blake, B. Weimer and H. Craven. Presentation at the annual Intermountain Conference of the American Society of Microbiologists (1996).
3. Production and isolation of heat-stable lipases from Pseudomonads. R. Koka, M. Hill, M. Blake, B. Weimer and H. Craven. Poster presentation at the national meeting of the American Dairy Science Association, Corvallis, Oregon (1996).
4. Heat-stability of Pseudomonad lipases. R. Koka, M. Hill, M. Blake, B. Weimer and H. Craven. Poster presentation at the Boneville meeting of the Institute of Food Technologists, Salt Lake City, Utah (1997).